Functional Regulation of the *Discs Large* Tumour Suppressor by Phosphorylation

Thesis

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Functional Regulation of the *Discs Large* Tumour Suppressor by Phosphorylation

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Contents

Abstract

The human homologue of the Drosophila Discs Large Tumour suppressor, hDlg, has been subject to various speculations concerning its role in the cell, with studies indicating roles in establishing and maintaining cellular polarity, as well as in controlling cell proliferation. The biochemical mechanism by which it might act in executing either function, have however remained ambiguous. In this study we show that phosphorylation is a major post-translational modification of the protein, affecting both location and function. We show that hDlg is phosphorylated both by the MAPKs and the CDKs, and both groups of kinases affect different aspects of the protein's behaviour. Post osmotic shock, the phosphorylation of hDlg by JNK leads to its accumulation in vesicular structures which we identify as endosomes, while its phosphorylation by the p38 MAPK in addition to relocating it to sites of cell-cell contact, also makes it more susceptible to degradation by the HPV18 E6 oncoprotein. Secondly, we show that hDlg is differentially regulated during the cell cycle, with each stage of the cell cycle leading to a different localisation of the protein, including its accumulation at the mitotic spindle in the M phase, as well as at the midbody during cytokinesis. We show also that the protein is phosphorylated by the Cyclin Dependent Kinases (CDK) 1 and 2, in a cell-cycle dependent manner on two sites - serine 158 and serine 442, and that these phosphorylations render the protein more stable and less susceptible to ubiquitination. Finally we show that hDlg phosphorylated on serine 158 and on serine 442 is largely nuclear, and that both the HPV18 E6 and the HPV16 E6 oncoproteins, target this nuclear form for degradation. These findings help us understand the processes that regulate hDlg and how these modifications of the protein might contribute to its growth-regulatory function in the cell.

Introduction

The Discs Large Protein - Domains and Structure

- The N terminus and L27 domain
- The PDZ domains
- The C terminus : The SH3, HOOK and GUK domains

The Epithelial Cell and Polarity

Dlg and Alternative Splicing

Protein Partners of Dlg

- Tumour Suppressors as interacting partners
- Viral proteins as interacting partners
- Other cellular partners

Dlg and Development

- Drosophila
- C.elegans
- Mice

Dlg and Phosphorylation

Abbreviations used

AJM-1 - Apical junction Molecule -1

AMPAR - alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

CASK - Calcium/calmodulin-dependent serine protein kinase

Dlg - Discs Large

hDlg - Human Discs Large

HPV - Human Papillomavirus

hscrib - Human Scribble

JNK - Jun N-terminal Kinase

MAGI - Membrane-Associated Guanylate kinase with Inverted orientation

MAPK - Mitogen Activated Protein Kinase

MDCK - Madine-Darby Canine Kidney cells

Net-1 - Neuro Epithelial Transforming Protein 1

PBM - PDZ Binding Motif

PDZ - Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg), and Zonula occludens-1 protein (Zo-1)

PML bodies - Pro Myelocytic Leukemia bodies

PTEN - (phosphatase and tensin homolog deleted on chromosome 10)

SAPK - Stress Activated Protein Kinase

The Discs Large (Dlg) Protein

The *Discs Large 1* (Dlg1) protein, also known as hDlg or sap97, is a representative member of a growing family of proteins collectively termed Membrane-Associated Guanylate kinase homologs (MAGUKs), whose other notable members include the ZO (Zonula Occludens) proteins - ZO-1, ZO-2 and ZO-3 and CASK (calcium/calmodulin-dependent serine protein kinase). It is the mammalian homologue of the *Drosophila* discs-large (Dlg), and has multiple protein domains encompassing three PDZ motifs, an L27 domain, an SH3 domain, and a Guanylate kinase-homology (GUK) domain (Craven and Bredt, 1998; Gonzalez-Mariscal et al, 2000). The dlg gene was originally identified as a tumor suppressor in Drosophila as recessive lethal mutations resulted in neoplastic overgrowth of imaginal disc epithelium (Stewart et al, 1972). Along with Scrib (Scribble) and Lgl (lethal Giant larvae), Dlg functions to control cellular proliferation in Drosophila and maintain cell integrity and polarity in both Drosophila and in mammals.

Drosophila Dlg has long been known to be involved in cell growth control, maintenance of cell adhesion and cell polarity in both embryonic and adult tissues and its inactivation results in the neoplastic growth of imaginal disc epithelial cells (Woods and Bryant, 1991; Woods and Bryant, 1993; Woods et al., 1996). Its closest human homologue is hDlg, and was originally cloned from B lymphocytes and was shown to be located on Chromosome 3 (Lue et al, 1994; Azim et al, 1995). Experimentally, Dlg was shown to have a tumour suppressive effect also in mammals. Thomas et al (1997) showed that the rat SAP97 functionally replaced Drosophila Dlg in a Dlg null background to prevent tumours. Massimi et al (2004) showed Dlg to have a tumour suppressive effect

in a cooperative transformation assay, where transfecting Dlg into primary baby rat kidney cells expressing EJ-ras and either HPV-16 E7 or adenovirus E1A suppressed the formation of colonies by inhibiting cell transformation. The existence of at least five alternatively spliced isoforms have been reported (Lue et al, 1994) and the presence of hdlg RNA transcripts are detected in a wide variety of human tissues.

The Discs large protein consists of a number of pivotal protein domains that mediate its interactions with a variety of important cellular and non-cellular proteins. It is a predicted 103kD protein, although it migrates as a doublet running at around 130kD on a western blot, suggesting the existence of intramolecular interactions within the protein, and posttranslational modifications, thereby giving rise to changes in conformation and migration. The hDlg protein contains a C-terminal domain homologous to the known guanylate kinases (GUK), a src homology 3 (SH3) region motif, a HOOK domain, an N-terminal L27 domain and three PDZ domains.

The N Terminus

In contrast to Drosophila Dlg, the targeting function of mammalian Dlg lies within the first 65 amino acids, which are absent in the Drosophila protein. These first 65 amino acids comprise an L27 domain which is widely reported as a protein-protein interaction domain that can assemble essential proteins for signalling and cell polarity into complexes by interacting in a heterodimeric manner (Li et al., 2004). Nakagawa et al showed that hDlg/ SAP97 L27 domain dimerisation with itself or its L27 interaction with other proteins was responsible for regulating synaptic delivery of AMPARs. In C. elegans, the L27 domain of DLG-1 mediates the physical interaction of DLG-1 with its binding partner, AJM-1, as well as DLG-1 multimerization (Lockwood et al, 2008). In mammalian epithelia, the PDZ1-2 and protein 4.1/HOOK domains provide

stabilization of the protein at the membrane (Wu et al., 1998). It has also been shown that the same amino-terminal domain found in mammalian Dlg is used by C. elegans Dlg-1 for protein targeting to adherens junctions (Firestein and Rongo, 200). The first 65 amino acids of hdlg, which are absent in other members of the family such as SAP90/PSD95 and SAP102, determine the subcellular localisation of the protein and provide at least one point of anchoring the protein to the cytoskeleton (Wu et al, 1998). The N terminal portion of Dlg also contains numerous Serines and Threonines, at least three of which have been reported to be phosphorylated by the SAPK/p38gamma under osmotic stress (Sabio et al, 2005). This portion of the protein thus assumes very important roles in determining the correct localisation of Dlg and is potentially important for the consequent regulation of the protein through post translational modifications.

The PDZ domains

PDZ (PSD-95/Dlg/ZO-1) domains are protein-protein interaction modules, 80 - 90 amino acids long, found on a large variety of cellular proteins. They have both structural as well as signaling functions. There are currently four different classes of PDZ interactions: recognition of carboxyl-terminal motifs in peptides, recognition of internal motifs in peptides, PDZ-PDZ dimerization, and recognition of lipids. Furthermore, PDZ domains that interact with C-terminal peptides can be divided into three separate classes according to their specificity (Cell Signaling Technology web literature: http://www.cellsignal.com/reference/domain/pdz.html ; Doyle et al., 1996)

Class Type	Motif	Example of PDZ protein	Example of binding partner
Class I	X - S/T - X- V/L	Dlg	HPV18 E6
Class II	X-Ψ-X-Ψ	CASK	Neurexin
Class III	X-D or E-X-Ψ	nNOS	Melatonin Receptor

The proteins containing PDZ domains use them to interact with other protein partners bearing a short carboxy-terminal PDZ binding motif (Kim 1997, Harris and Lim 2001). hDlg is a class I type PDZ protein that has three structurally clustered PDZ domains and specific PDZ domains of hDlg have been shown to interact with the C-termini of several proteins, including the mitotic kinase TOPK/PBK (Gaudet et al., 2000), the human papillomavirus (HPV) E6 oncoprotein (Kiyono et al., 1997; Lee et al., 1997), the HTLV-1 Tax oncoprotein (Suzuki et al., 1999), the HTLV-1 Env protein (Blot et al, 2004), the Adenoviral E4 ORF1 (Lee et al., 1997) and APC (Matsumine et al., 1996) (see figure 1). Marfatia et al demonstrated that the organization of the PDZ domains on hDlg is in two conformationally stable modules - one comprising PDZ domains 1 and 2, while the other consists of PDZ domain 3. They show that the former, the module comprising PDZ 1+2, has a high affinity for the cytoplasmic domain of shaker-type K+ channels and specifically binds ATP, implicating PDZ-domain mediated functions for Dlg in membrane clustering of the shaker-type channels and in cytoskeletal interactions (Tejedor et al., 1997).

Affinity interaction experiments with GST-fusion proteins containing the modular domains of SAP97 demonstrated that the second PDZ domain is sufficient for interaction with the C terminus of the potassium channel Kir2.2 in the brain and this association may form part of a macromolecular signalling complex in many different tissues (Leonoudakis et al, 2001).

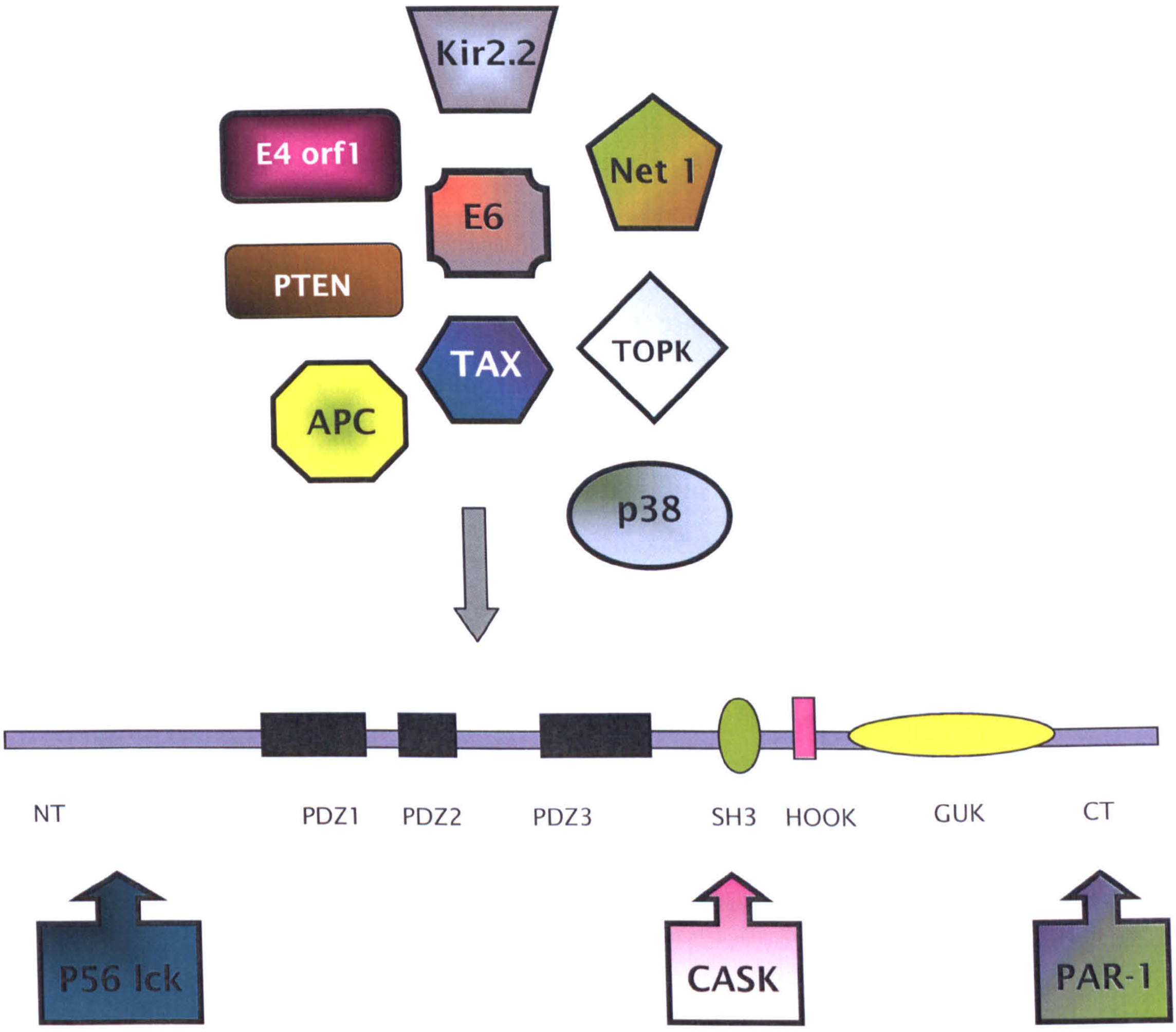


Figure 1: The Dlg Protein. A schematic of the Dlg protein with its important protein interaction domains and interacting partners.

The C Terminus: The SH3, HOOK and GUK domains

Postsynaptic targeting of Drosophila Dlg critically depends on its SH3 and GUK domains. In contrast, a recently described mouse mutant lacking the SH3 and GUK domains of SAP97 neither changes Dlg's subcellular distribution nor does it disrupt synaptic structure or protein clustering although these mice had very acute developmental defects (Klocker et al, 2002). This also suggests that the regulation of Dlg localisation in different organisms is most likely controlled by different mechanisms, and great care therefore needs to be taken in assigning functions across different species. In C.elegans, the inclusion of the SH3 domain could ameliorate dlg-1 mutant phenotypes. However, full rescue of lethality required the complete C terminus, which includes the GUK and HOOK domains, thereby demonstrating the importance of the C-terminus for DLG-1 function (Lockwood et al, 2008). The phosphorylation of Dlg by the PAR-1 Kinase is on Serine 797 in the C-terminus is a critical event in synaptogenesis as this results in negative regulation of the mobility of Dlg and its targeting to the synapse (Zhang et al, 2007). The 34-aa long HOOK domain of the hDlg protein lies between the SH3 and the GUK domains of alternately spliced isoforms of hDlg bearing the I3 insertion (Lue et al., 1994). This region has been shown to bind protein 4.1 (Lue et al., 1994), a major structural element of the erythrocyte cytoskeleton which regulates membrane physical properties and is located at the septate junction. This interaction might explain the different localisations and subsequently different functions, of the different isoforms of hDlg in the cell.

The Epithelial Cell and Polarity

Epithelial cells line the cavities and surfaces of structures throughout the body, including the insides of the lungs, the heart and the urinary tract as well

as the outer surface of the cornea (*Molecular Biology of the Cell*, 4th edition, Alberts et al., 2002). Epithelial cells exist as monolayers where cells are packed closely together and the structural integrity of the cell is emphasized by the presence of junctions. A typical vertebrate epithelial cell has four types of junctions: Tight junctions, Adherens junctions, Gap junctions and Desmosomes (*Molecular Biology of the Cell*, 4th edition, Alberts et al., 2002). Tight junctions or Zonula occludens consist of the transmembrane proteins occludin and claudin (Hartsock and Nelson, 2008). They are found in the apical region of the cell and function in preventing the passage of molecules and ions in the spaces between cells as well as blocking the movement of integral proteins between the apical and the basolateral surfaces of the cell. Adherens junctions or Zonula Adherens are protein complexes that occur more basal to the tight junctions, and are composed of proteins like the cadherins and the catenins (Yap et al., 1997). Vertebrate homologues of Dlg, along with Scribble and Lgl are found to localize to the basolateral region and the adherens junctions, but not the tight junctions, whereas in Drosophila, Dlg and Scribble are found at the septate junctions which are located below the adherens junctions (Yamanaka and Ohno, 2008). Together, this tripartite complex is considered essential for the establishment of basolateral polarity in epithelial cells. Bellaiche et al (2001) showed the involvement of Dlg also in establishing planar cell polarity in the sensory organ precursor cells in Drosophila, where it forms a complex with Pins and this complex responds to Frizzled (Fz) signaling thereby creating planar asymmetry in the cell.

Loss of cell polarity is now established as one of the most commonly observed events in the advanced stages of cancer, along with loss of cell-cell adhesion and consequent loss of tissue architecture, leading to tissue invasion and metastases. Initial studies on Lgl and Dlg showed that individual mutations in both genes lead to the overgrowth of imaginal discs in Drosophila larvae and

to malformations in the apical junctions (AJs). The pivotal study that linked the Drosophila tumour suppressor genes Scrib, Dlg and Lgl together came in 2000 from Bilder et al. They showed that Scrib, Dlg and Lgl act in concert to maintain epithelial cell structure and regulate cell proliferation in Drosophila (Bilder et al., 2000). Zygotic mutations in any of the three genes led to similar defects – disorganization and overgrowth of epithelial cells in larval imaginal discs, disruption of AJs in the embryo and polarity defects in follicle cells. Further, they also demonstrated that in the embryonic epidermis, cortical Scrib or Lgl localization depends on Dlg, whereas the localization of Dlg to Septate Junctions requires both Scrib and Lgl. These set of experiments definitively prove that Scrib, Dlg and Lgl interact together genetically in Drosophila to regulate a common pathway that controls cell polarity and proliferation.

Additionally, Ohshiro et al., (2000) demonstrated the essential role of tumour suppressor proteins Lgl and Dlg in linking cortical polarity to asymmetric cell division. Normally Drosophila is an excellent model in which to study asymmetric cell division, as it exhibits establishment of distinct apical-basal cortical domains, an asymmetric mitotic spindle aligned along the apical-basal axis, and the neuroblast divides unequally to produce a large apical neuroblast and a small basal daughter cell. The study underlined the importance of Lgl in the asymmetric cortical localization of basal determinants in mitotic Drosophila neuroblasts, making it indispensable for neural fate decisions. The function of Lgl was shown to be aided by the role of Dlg in regulating the localization of Lgl. Thus, Lgl and Dlg act in a common process that differentially mediates cortical protein targeting in mitotic neuroblasts, thereby creating intrinsic differences between daughter cells.

A later study by Albertson et al., (2003) supported this further by showing that the three proteins together can regulate several aspects of

asymmetric cell division in Drosophila neuroblasts. Dlg/Scrib/Lgl mutants show marked defects in apical cell and spindle pole size and lead to symmetric or inverted neuroblast cell divisions. These two pivotal papers corroborate the role of Dlg, Scrib and Lgl in linking cortical polarity with asymmetry in cell size and mitotic spindle in Drosophila neuroblasts.

Scrib, a scaffolding protein which belongs to the LAP (LRR (leucine rich repeats) and PDZ domain) family of proteins, has 16 leucine rich repeats and 4 PDZ domains. It is a protein involved in the regulation of cell adhesion, cell shape and polarity (Bilder et al., 2000). In Drosophila, the LRR tethers Scrib to the plasma membrane and is necessary and sufficient to organize a polarized epithelial monolayer by controlling apicobasal polarity and consequently proliferation. Although the PDZ domains recruit the LRR to the junctional complex and the absence of the PDZ domains leads to mild polarity defects and moderate overproliferation, the PDZ domains alone are insufficient to provide any Scrib function in mutant discs (Zeitler et al., 2004).

Lgl is a cytoskeletal protein belonging to the WD repeat L(2)GL family of proteins which are characterised by the presence of WD (tryptophan-aspartate) repeats. Lgl has two WD-40 repeats which are involved in protein-protein interactions; loss of Lgl leads to massive tissue disorganization, tumor-like growth and lethal phenotypes in both Drosophila and mice (Humbert et al., 2003). Lgl mutant cells display disruption of cell polarity, failure of asymmetric cell division, deregulation of Notch signaling and loss of proper cell fate determination (Vasioukhin, 2006).

In the mammalian context, a few notable studies have highlighted the role of the triple complex in the maintenance of cell polarity as well as cell proliferation. Thomas et al (1997) heterologously expressed rat SAP97 in

Drosophila to test for functional homology and found that it suppresses tumour formation in dlg-1 mutant flies. Dow et al (2003) showed that the Scribble human homologue, hScrib when expressed in a scrib mutant Drosophila background, could rescue the polarity and cellular overgrowth defects, demonstrating that the human homologue can functionally replace the drosophila form in vivo. Dow et al (2007) have also shown an involvement for Scrib alongwith the Par polarity complex in the regulation of cell migration. A similar study by Grifoni et al (2004) showed the same behavior also for Hugl-1, the human homologue of Lgl, which could rescue larval lethality and prevent neoplastic features in Drosophila Lgl homozygous mutants. Taken together, all these studies suggest that the genetic pathway comprising the tumour suppressors Lgl, Dlg and scrib is in part conserved from Drosophila to mammals.

Takizawa et al (2006) also described an interaction between the PDZ1 and PDZ4 domains of hScrib with the C terminal PDZ binding motif of APC, in much the same way as hDlg interacts with APC (Matsumine et al., 1996). In this case, the presence of hScrib was deemed necessary for the correct localisation of APC at the adherens junction. The data also suggest that hScrib may participate with hDlg and APC in the complex regulating the cell cycle. Interestingly hScrib is also targeted by the viral oncoprotein high risk HPV E6 for degradation (Nakagawa et al., 2000; Massimi et al., 2004) and is mislocalised by the HTLV-1 Tax oncoprotein (Arpin-Andre and Mesnard, 2007). Both these oncoproteins also target hDlg, implicating the concerted effect of these cellular polarity proteins in maintaining the integrity of the cell and controlling cellular proliferation.

Alternative splicing and the isoforms of Dlg

The initial cloning of hDlg from lymphocytes also led to the discovery that the sequence undergoes alternative splicing to give rise to at least five different isoforms (Lue et al, 1994; McLaughlin et al., 2002). These additional isoforms arise as a consequence of different transcription start points and at least five different hDlg gene products are predicted. The isoforms are referred to as the I1, I2, I3 and I5, and as Figure 2 illustrates, only the proline rich I1 isoforms are located in the N terminus, while the rest of the splicing events generating the other isoforms are found in the C terminal regions of the protein. Isoforms I2 and I3 are mutually exclusive of each other while isoform I5 is found between the I3/I2 and I4 insertion sites. Further characterisation of the function of these isoforms came from McLaughlin et al (2002), where they not only identified a new insertion N-terminal to the PDZ regions called I1B, but also determined that the insertions in the second region were responsible for influencing the localisation of hDlg. Thus, the I3 insertion targets the protein to cell-cell contacts, presumably through binding 4.1 (Lue et al, 1994; 1996), while the I2 insertion targets the protein to the nucleus. PCR analysis performed on cDNA molecules derived from human tissues found I3, I2, and I5 to be present in brain, placenta, skeletal muscle, kidney, liver, cardiac muscle, lung, and placenta. In contrast, the I4 isoform was detected only in brain and liver, and in both tissues the I2, I4 and I5 combination was predominantly expressed. Alternative splicing of the hDlg gene also has a brain-specific variant expression pattern, where the form without the I1 insertion was found to be predominantly expressed (Mori et al, 1998). Changes were also observed in the splicing patterns between normal brain and neuroblastoma cell lines, signifying a role for selective or specific splicing in malignant tissue. A recent study by Roberts et al (2007), also proposed a role

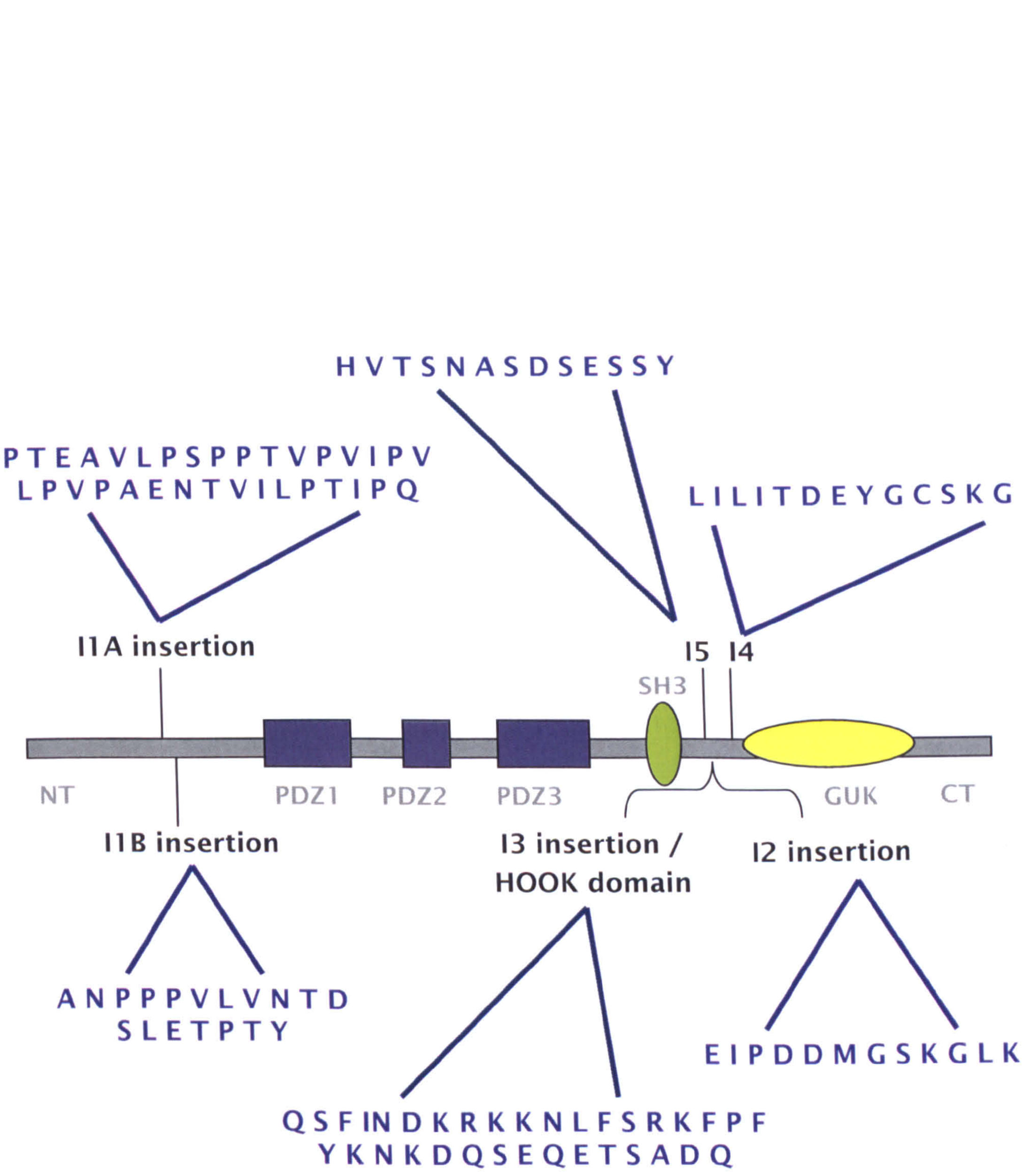


Figure 2 : The different isoforms of Dlg (see text for details)

for the various isoforms in the different stages of epithelial differentiation. They observed an overall dynamic redistribution of hDlg with the exit of the I2 isoform from the nucleus when epithelial differentiation was stimulated, and a concomitant massive increase in the expression of the I3 isoform accompanied by increased membrane staining and a moderate decrease in the levels of expression of the I2 isoform. The correlation between the redistribution of the variants and the change in differentiation stage suggests that the two processes might be linked.

Protein Partners of Dlg

Tumour Suppressors as interacting partners

APC

The most intriguing account of hDlg binding another cellular protein is conceivably its interaction with the APC tumour suppressor protein. This was first described by Matsumine et al (1996), when they found a positive interaction between the PDZ binding motif in the carboxy terminal region of APC, and a portion of hDlg encompassing amino acids 199 to 507, in a two hybrid screen of a human brain cDNA library. Dlg forms a complex with APC through APC's C-terminal PBM and the PDZ-2 domain of Dlg. They then confirmed this interaction with GST binding and co-immunoprecipitation assays. Immunofluorescence analysis in rat hippocampal neurons and colon epithelial cells showed a similar pattern of distribution for both proteins and a high degree of colocalisation along the lateral plasma membrane. Interestingly, although β-catenin is not known to interact with hDlg directly, it was also shown to coimmunoprecipitate with hDlg and APC implying that the three proteins are part of the same complex in vivo. The APC-Dlg complex has also been shown to negatively regulate the cell cycle from the G0/G1 to the S phase (Ishidate et al, 2000). Although the APC-Dlg interaction implies a role in cell

cycle regulation, a more recent study has also linked it to the regulation of cell polarity. Using total internal reflection fluorescence (TIRF) microscopy - an elegant optical technique used to observe single molecule fluorescence at surfaces and the interaction of molecules with surfaces - and immunoprecipitation techniques, Etienne-Manneville et al (2005) showed that Cdc42 and the Par6-PKCzeta complex regulate the spatial association of Dlg and APC, and this physical interaction between them is necessary not only for the polarization of the microtubule cytoskeleton, but also for centrosome reorientation and cell migration. APC clusters have also been shown to colocalise with Dlg at cellular protrusions of subconfluent MDCK cells, and although the disruption of microtubules by nocodazole abolished this interaction, the depolymerisation of actin filaments with latrunculin A did not (Iizuka-Kogo et al, 2005), suggesting that this interaction is microtubule mediated.

E-Cadherin

The role of E-cadherin as a tumour suppressor has long been established. It is involved as a cell-cell adhesion molecule in epithelial cells - from which 80% of all cancers arise - and has fundamental roles in the later stages of tumor progression (reviewed in Semb and Christofori, 1998). Dlg/SAP97 has also been shown to associate with an E-cadherin/β-catenin complex. However, direct binding of hDlg to the components of this complex has not been detected. Association with this complex is believed to occur indirectly through the attachment of Dlg/SAP97 to the cortical cytoskeleton which is assembled by E-cadherin mediated cell-cell adhesion (Reuver and Garner, 1998). Laprise et al (2004) reported the colocalisation of hdlg with E-cadherin at sites of cell-cell contact in intestinal epithelial cells. Further, RNAi to hdlg in these cells changes the integrity of adherence junctions and prevents the recruitment of phosphatidylinositol 3-kinase (PI3K) to E-cadherin mediated cell-cell contact

sites. They also reported that hdlg, E-cadherin and PI3K exist in a macromolecular complex which requires the association of Dlg with the Src Homology 2 (SH2) domain of the PI3K subunit. In the mouse eye, Dlg-1 and Scribble colocalise with the E- and N-cadherins suggesting an important role for these interactions in the regulation of cell proliferation, adhesion and polarity. These studies also a highlight a high degree of functional conservation across species (Nguyen et al, 2005).

PTEN

An interaction between hDlg and the PTEN tumour suppressor has also been demonstrated (Adey et al., 2000). PTEN (phosphatase and tensin homolog deleted on chromosome 10), is a phosphatase that can dephosphorylate tyrosine-containing peptides, Shc, focal adhesion kinase and phosphoinositide substrates and increasing evidence points towards this activity of PTEN as being responsible for its tumour suppressive activity. On performing two-hybrid searches with PTEN, hDlg was identified as a binding partner. Further two-hybrid analyses and microtiter plate binding assays showed that the sites of interaction between the proteins were the PDZ domains of Dlg and the C - terminal PDZ binding motif of PTEN. Threonine phosphorylation of the PTEN-PDZ Binding Domain peptide inhibited binding to hDlg (Adey et al., 2000). A C-terminal deletion of PTEN does not affect its phosphatase function and is still active in downregulating PKB, but this mutant enzyme had no effect on platelet-derived growth factor (PDGF)-induced membrane ruffling and was only partially active in a cell viability assay. (Leslie et al, 2000 and 2001). Whether this is a direct consequence of PTEN's inability to bind hDlg remains to be seen. Interaction of PTEN has also been seen with other MAGUK members such as the MAGI proteins (Wu et al., 2000), with MAGI-2 in particular showing inhibition of cell migration in hepatocarcinoma cell lines when associated with PTEN (Hu et

al., 2007). These studies imply a conserved and putatively important role for the interaction of PTEN with PDZ domain containing proteins.

Viral oncoproteins as interacting partners

Cancer induced by viruses account for 15% of all human cancers (Gatza et al, 2005). Perhaps the most documented role of Dlg in cancer is its role in Human Papilloma Virus (HPV) mediated cervical cancers. Human Papillomaviruses are small double stranded DNA viruses which cause a plethora of neoplastic diseases of the epithelia, stretching from benign warts to lethal carcinomas. Cervical cancer caused by HPV is the second major cause of cancer-related death in women worldwide (Parkin, 2006), and HPV infection has been detected in virtually 100% of all cervical cancer cases. The virus is also capable of infecting other epithelial sites and is therefore linked to a number of clinical pathologies including benign warts of the skin and a number of head and neck cancers (Gillison and Shah, 2001). Only a small subset of the many HPV types known are associated with the actual development of cancer (de Villiers, 1994) and are accordingly classified into high-risk or low-risk types. The high risk types which are connected to cervical cancer are HPV-16, 18, 31 and 33 (zur Hausen, 1991). Their low risk equivalents, such as HPV-6 and 11, also infect the mucosal epithelia and cause the growth of genital warts; however these are rarely associated with malignant lesions.

Initial infection of Papillomaviruses probably occurs through microtraumas in the upper layers of the epithelia which provide the virus with access to lower basal cells. In these cells, viral episomes are maintained at a low copy number of between 10-200 copies per cell (De Geest et al., 1993). In the infected basal cells, the early protein E2 plays a primary role in viral genome replication by recruiting the viral helicase, another early protein

called E1, onto the origin of replication (mohr et al., 1990). As the infected basal cell layers start to differentiate, their replicative capacity is greatly reduced (Watt et al., 2006), and the viral episomes are maintained at high copy number. A replication switch to rolling circle mode is observed (Flores et al., 2000) and the E6 promoter is activated, resulting in the enhanced expression of the early proteins E1, E2, E5, E6 and E7. The gene products of three early genes, E5, E6 and E7, possess proliferation stimulatory effects, though E5 only has a subtle role in the productive stages of human papillomavirus infection. Both high and low risk types are highly successful viruses; however there is a massive difference in their transforming potential. This difference arises not because of major sequence variations between the two sets of viruses, but rather due to subtle biochemical and biological differences in the functioning of the major oncoproteins E6 and E7. The oncogenic potential of the high-risk types is dependent on the cooperative action of these two proteins, which bind and alter the activity of cell cycle regulatory proteins. Expression of E6 and E7 in concert can induce a variety of tumours, depending on the tissue type in which it is targeted (Griep et al 1993, Comerford et al 1995). In this context, the partnerships and interaction of these major oncoproteins with other cellular proteins becomes very essential.

The HPV E6 protein has long been acknowledged as a potent oncogene and is one of the key players in the events that lead to malignancy in virally infected cells. It interacts with a number of cellular proteins, the most investigated of which is its interaction with the tumour suppressor p53. E6 targets p53 for proteasome-mediated degradation by binding to a HECT domain-containing ubiquitin ligase, named E6-Associated protein (E6-AP) (Scheffner et al 1993). Viral genomes which have a disruption of E6 expression or have mutations in the region of E6 that binds p53 fail to maintain viral episomes (Park and Androphy, 2002; Thomas et al, 1999). Additionally it

functions as a transcriptional modulator by interacting with the p300/CBP transcriptional co-activators (Patel et al 1999, Zimmermann et al, 1999, 2000), disrupts cell junctions (Mantovani et al, 2001) and suppresses apoptosis by hindering cellular proteins such as Bak (Thomas and Banks, 1998), Bax (Li and Dou, 2000), Fas (Filippova et al, 2004) and c-myc (Gross-Mesilaty et al 1998). It is a protein comprising 150 amino acids, with a hydrophilic N-terminus followed by the presence of two zinc fingers formed by Cys-X-X-Cys motifs (Cole and Danos, 1987; Barbosa et al., 1989). These zinc finger domains are essential for practically all the biological functions of the E6 protein (Kanda et al, 1991) and they are highly conserved throughout all HPV types. The high risk mucosal HPV E6 proteins are unique in possessing a PDZ domain-binding motif (PBM) at their extreme carboxyl-termini (Kiyono et al, 1997, Lee et al, 1997), which is not involved in p53 binding and degradation (Pim et al 1994). Interestingly, the low risk HPV E6 proteins lack the PBMs (Thomas et al 2001; table 1).

TABLE 1 : Various HPV E6 types and their PBMs.

Virus Type	Sequence (- *X -T/S- X-V*)
HIGH RISK	
HPV-16E6	CC.....RSSRTRR*ETQL*
HPV-18E6	CCNRARQERLQRRR*ETQV*
HPV-45E6	CCDQARQERLRRRR*ETQV*
HPV-31E6	CW.....R.RPRT*ETQV*
HPV-33E6	CW.....R.SRRR*ETAL*
HPV-35E6	CW.....K.PTRR*ETEV*
HPV-56E6	CW.....RQTS.REPR*ESTV*
HPV-66E6	CW.....RHTS.RQAT*ESTV*

LOW RISK	
HPV-6E6	CWTTCMEDMLP.....
HPV-11E6	CWTTCMEDLLP.....
HPV-42E6	CRGQCVERRLP.....
HPV-44E6	CWTSCMETILP.....

Dlg was the first PDZ-protein substrate shown to bind the C terminal PBMs of high risk HPV-16 and HPV-18 E6 proteins (Kiyono et al 1997; Lee et al, 1997). E6 then targets Dlg for ubiquitin mediated degradation (Gardiol et al 1999, Kuhne et al 2000), either by itself or by accelerating the physiological turnover of cellular Dlg, as Dlg is ubiquitinated and degraded by the proteasome also in the absence of E6 (Gardiol et al, 1999; Mantovani et al, 2001). E6 also interacts with other PDZ-domain containing substrates which are involved in the establishment and maintenance of cell polarity such as Scribble (Nakagawa and Huibregtse, 2000) and the MAGI proteins (Glausinger et al, 2000; Thomas et al 2002). Moreover, the interaction is highly type-specific, in that changing a single amino acid in the PBM affects the binding specificity of the HPV type to its PDZ domain containing substrate, with HPV-18 E6 showing higher specificity to bind and degrade Dlg than HPV-16 (Thomas et al, 2001). An additional mechanism that regulates the interaction of the HPV E6 with Dlg, is the phosphorylation of E6. Phosphorylation of E6 at its PBM by Protein Kinase A (PKA) negatively regulates its ability to interact with Dlg, but doesn't affect its ability to degrade p53 (Kuhne et al, 2000; Massimi et al, 2001). Unlike p53, the degradation of Dlg by E6 is not E6-AP dependent (Grm et al, 2004; Pim et al, 2000), and suggests the involvement of other as yet unknown ubiquitin ligases.

Although at this stage it is impossible to state ultimately how critical the Dlg interaction is for HPV induced malignancy, there is compelling evidence that highlights its importance. Transgenic mice expressing an E6 lacking the last six amino acids, fail to show epithelial hyperplasia (Nguyen et al, 2003), and fail to contribute to the promotional stages of skin carcinogenesis experiments, although they retained their ability to take part in progression (Simonson et al, 2005). These studies obviously point to a vital role for the PDZ binding motif. Analysis of Dlg in histological specimens from patients with HPV-positive invasive carcinomas showed not only a marked reduction in Dlg levels, but also massive alterations in localisation of the protein in cells and tissues as the lesions developed (Watson et al, 2002; Cavatorta et al, 2004).

Other viral proteins that bind Dlg and interfere with its cellular functions are the HTLV-1 Tax Oncoprotein and the HTLV-1 Env glycoprotein (Suzuki et al., 1999, Blot et al, 2004), and the Adenovirus 9 E4 ORF1 proteins (Lee et al 1997). The HTLV-1 Tax also possesses the C-terminal PBM and binds Dlg in vitro (Lee et al, 1997), leading to perturbation of the negative cell cycle regulatory function of Dlg (Suzuki et al, 1999). Dlg was also identified as a binding partner also for the Env protein in a yeast two-hybrid assay and was shown to interact with the PBM on the C-terminus of Env through its PDZ domains, and this seems to be critical for Env mediated cell fusion as a mutant virus unable to bind Dlg exhibited a decreased ability to trigger fusion events (Blot et al, 2004). Whether this has any role in HTLV- related tumour promotion is unclear. However, it is compelling that the PBM-containing HTLV-1 Tax protein shows a strong correlation with development of adult T cell leukemia, while the HTLV-2 Tax protein that has not been reported to be associated with malignant leukemias lacks the PBM (Hirata et al., 2004; Tsubata et al., 2005).

The oncogenic potential of the adenovirus E4-ORF1 protein correlates strongly with its binding to Dlg as well as other cellular PDZ proteins such as MUPP1, MAGI-1 and ZO-2 via the C terminal PBM. Recent studies have however shown, that on binding to the adenovirus E4ORF1, Dlg1 containing the I3 isoform may also have an oncogenic potential by activating the phosphatidylinositol 3-kinase (PI3K) pathway. Frese et al (2006) showed that Dlg1$^{-/-}$ mouse embryo fibroblasts (MEFs) are not able to support E4-ORF1 induced PI3K activation and cellular transformation. Also, activation of PI3K by growth factors remained normal in these Dlg1$^{-/-}$ MEFs, revealing a Dlg-dependent defect in E4-ORF1-induced PI3K activation. Though both E4ORF1 and Tax-1 bind Dlg, they do not target it for degradation indicating, as evidenced by the study above, that the interaction between the Dlg and the respective oncoproteins might give rise to a functional complex. The activities of all three viral oncoproteins acknowledged here in the context of Dlg, pertain at least in part, to disrupting the complex formed between the C-terminal PBM of tumour suppressors such as APC, PTEN and the PDZ domains of Dlg by competing for the interaction with these cellular tumour suppressors, and consequently abrogating the negative cell-cycle regulatory function of the complexes.

Other Important Cellular Partners

The cytoskeletal protein 4.1 was probably the first reported binding partner of hdlg (Lue et al, 1994), with the interaction occuring through the N-terminal 30 amino acid region of 4.1 and the HOOK region of hdlg, thereby localizing the complex to cell junctions. The alternatively spliced I3 domain-mediated targeting of hDlg depends exclusively on interactions with 4.1/ERM

proteins (Lue et al, 1996). In addition to the N-terminal targeting domain, the alternatively spliced I3 version of hDlg also plays an important role in recruiting the protein to the lateral membrane in epithelial cells via its interaction with protein 4.1 (Hanada et al, 2003). The membrane bound localisation and the translocation of hDlg thereafter to the midbody at cytokinesis are processes that are independent of its binding to 4.1 (Massimi et al, 2003). Strikingly, this complex seems to be of great relevance to development in mice, as mice expressing a mutant form of Dlg lacking the SH3, HOOK and GUK domains show severe growth retardation and die perinatally (Caruana and Bernstein, 2001), although the high probability of these mutations affecting other equally strong interactions cannot be ruled out.

GKAP or Guanylate Kinase-Associated Protein is a synaptic protein that binds all members of the mammalian PSD-95 family. The C-terminal GUK domain of Dlg shares a high degree of sequence similarity with low-molecular-mass guanylate kinases and though it was therefore implicated in signalling events, it was found that it did not encode for an active guanylate kinase and that binding GKAP does not confer any of these enzymatic properties upon it either (Kuhlendahl et al, 1998). However, the binding of GKAP to Dlg is strictly regulated by a series of intramolecular interactions, involving conformational changes in Dlg caused by the interface between the SH3 and the GUK regions which interfere with binding to GKAP, while an N terminal interaction with the SH3 facilitates the inter-protein interaction. This sheds a lot of light on the communication between Dlg and its domains and how these events determine binding partners for the protein (Wu et al, 2000). The mechanism that allows the GKAP-Dlg contact to occur came from work by Sabio et al., (2005), when they showed that the SAPK/p38 gamma mediated phosphorylation causes its prompt dissociation from GKAP and from the cytoskeleton in response to osmotic stress.

CASK is another interacting PDZ domain-containing protein partner that also belongs to the MAGUK family. Nix et al (2000) showed the interaction of the human homologue hCASK, with hDlg through colocalisation in immunofluorescence at basolateral membranes of epithelial cells in small and large intestine and co-precipitations from lysates of the intestinal cell line, Caco-2. They demonstrated the binding of the GUK domain of hCASK to the SH3 domain of hDlg, and implicated this functional complex in MAGUK scaffold assembly. Sanford et al., (2004) showed that CASK and Dlg are present together at the mammalian neuromuscular junction in skeletal muscle, suggesting that they may act together as an organizing scaffold at the postsynaptic junction. A macromolecular complex consisting of CASK, Dlg/SAP97, Veli and Mint1 associate with the kir2.2 potassium channel via kir2.2's C-terminal PBM to mediate plasma membrane localisation and trafficking (Leonoudakis et al, 2004). The association of Dlg with CASK also seems to be crucial for lateral localization of SAP97 in MDCK cells (Lee et al., 2002).

A recent study (Garcia-Mata et al., 2007) demonstrated an interaction between Dlg and Net1 - a RhoA-specific guanine nucleotide exchange factor localized in the nucleus, which acts as an oncogene when it has a deletion in the N terminus. Net1 contains a PBM in its C-terminus through which it interacts with the PDZ domains of Dlg and other members of the Dlg family, and promotes the translocation of the PDZ proteins to nuclear subdomains associated with PML bodies. The oncogenic mutant however loses the ability to shuttle Dlg to the nucleus, implying that when oncogenic, it sequesters Dlg in the cytosol and prevents it from carrying out its normal cellular function, thereby resulting in Net1-mediated transformation. This interaction seems important in shedding light on the functional aspects of the nuclear component of hDlg and on the importance of the interaction of Dlg with specific cellular partners in the progression of neoplastic disease.

Dlg and Development

Drosophila

The role of Dlg in development seems to be a very critical one. In Drosophila, Larvae missing zygotic l(1)dlg-1+ gene activity die due to aberrant growth of imaginal cells at the larval-pupal transition, whereas embryos lacking both maternal and zygotic activity of l(1)dlg-1+, show neurogenesis and morphogenesis defects that result in very abnormal embryos (Perrimon, 1988). The localisation of Dlg in the periactive zones of the Drosophila neuromuscular synapses defines its role in synapse development (Sone et al., 2000). Dlg is also required for normal synaptic structure in the brain (Lahey et al., 1994). The implication of Drosophila Dlg in developmental pathways is shown through the change in shape of follicle cells during oogenesis and their ability to invade when there is a loss of Dlg activity (Goode et al., 1997).

Furthermore, each domain of the Dlg protein has a different function in development and in the organisation of a functional junctional complex - the HOOK region decides the membrane bound localisation of Dlg, its postsynaptic targeting and its role in epithelial structure, the GUK domain routes Dlg between compartments, while the PDZ2 and PDZ3 regions seem to control growth regulation (Hough et al., 1997; Thomas et al., 2000).

Studies also indicate that Dlg along with Scribble and Lgl acts together in a common pathway to regulate cell polarity and growth control (Bilder et al., 2000). In Drosophila, Lgl has also been shown to be indispensable for neural fate decisions, and Dlg participates in this process by regulating the localisation of Lgl (Ohshiro et al., 2000). During embryonic development, the isoforms of Dlg are principal in regulating neuronal differentiation and organization. These

isoforms are expressed at larval neuromuscular junctions and within the CNS of both embryos and larvae but not detected in epithelial tissues, showing a domain and isoform-specific role of Dlg in Drosophila neuronal development (Mendoza et al., 2003). Additionally, embryos carrying mutations in either Dlg or the integral membrane protein Strabismus (Stbm) showed severe imperfections in plasma membrane formation, while overexpression of either component induced excessive plasma membrane formation (Lee et al., 2003). The functional genetic complex formed between Dlg and Stbm is essential for the formation of new plasma membrane. Mendoza-Topaz et al., (2008) recently unveiled the differential roles in neural development of the two forms of the Drosophila MAGUK gene Dlg - DlgA (PSD95 (postsynaptic density 95)-like) and DLGS97 (SAP97 (synapse-associated protein)-like). Generation of independent null mutations in both showed predominant expression of DLGA in the embryo, balanced expression of both during larval stages, and almost exclusive DLGS97 expression in the adult brain.

C. elegans

Dlg-1, the C. elegans homologue of the Drosophila Dlg protein, is essential to Zonula Adherens junction formation in the organism, independently of the cadherin-catenin system and nematodes knocked down for Dlg-1 function arrest as two-fold stage embryos and show abnormalities in the organisation of the actin cytoskeleton (Bossinger et al., 2001; Firestein and Rongo, 2001). Apical junction molecule-1 (AJM-1) is an important protein that determines the integrity of the apical-junctional domain in C. Elegans. The proper localisation of this protein depends on Dlg-1 and Let-413, without which AJM-1 is completely lost from apical junctions in embryos (Koppen et al., 2001).

An RNAi screen against C. elegans homologues of genes encoding proteins involved in tight or septate junction formation identified Dlg-1 as an essential gene, and Dlg-1 is required to cause aggregation of the JAM-1 and other proteins involved in maintaining the C. Elegans Apical Junction (CeAJ) (McMahon et al., 2001). Also, the C. Elegans cell junction protein Vab9, when mutated enhanced adhesion defects due to the loss of Dlg-1 and AJM-1 function (Simske et al., 2003). A recent study in C. elegans has delineated the importance of the various domains of Dlg-1, specially the entire C - terminus which seems essential to rescue the lethal phenotype of a Dlg-1 mutant (Lockwood et al., 2008).

Mice

The role of Dlg in development has also been demonstrated in mice. The interaction of the Adenomatous Polyposis Coli (APC) protein with Dlg has a role not only in controlling tumorigenesis, but also in development. Mice carrying APC with a homozygous mutation for a missing carboxy-terminal domain enter postnatal life, but show growth retardation, reduced viability on the B6 genetic background, missing preputial glands, and the formation of nipple-associated cysts (Smits et al., 1999). Mutations resulting in a truncated version of Dlg have shown to impair morphogenesis during murine development and results in perinatal death (Caruana and Bernstein, 2001). Nguyen et al (2003) used wildtype and C-terminal mutant versions of the HPV E6 oncoprotein in the mouse ocular lens to discern the role that Dlg along with the other major PDZ-containing tumour suppressors, Scrib and the MAGIs, might play in epithelial cell growth and differentiation. They found these proteins to be intimately linked with cell cycle regulation, cell adhesion and structure. Mice expressing a truncated version of Dlg1, lacking the SH3, HOOK and GUK domains showed disruption of epithelial polarity and abnormalities in nephrogenesis (Naim et al.,

2005). Mahoney et al., (2006) generated a null allele of Dlgh1(Discs-large homolog-1) to study its role in urogenital development and found that the mice developed several abnormalities including congenital hydronephrosis, misalignment of smooth muscle cells and severely impaired ureteric peristalsis, suggesting that Dlgh1 also regulates smooth muscle orientation and mutations in human Dlg1 may contribute to hereditary forms of hydronephrosis. Another report documenting a Dlgh1 knockout apart from observing defects in the renal and urogenital organs, also suggested a novel role for it in regulating epithelial duct formation and morphogenesis during mammalian development (Iizuka-Kogo et al., 2007).

Dlg and Phosphorylation

Phosphorylation is a very important post-translational modification which regulates numerous courses of action in the life of a cell. It is intimately linked to protein function in several biological pathways, such as the cell cycle, protein ubiquitination and degradation, signal transduction networks and many phosphorylation events are critical to cell survival. It is estimated that 50% of the proteins in the proteome are phosphorylated. Phosphorylation often occurs on multiple distinct sites on a given protein with phosphorylation on serines being most commonly seen, followed by threonines and relatively less on tyrosine residues.

Dlg is a serine and threonine rich protein, with over 80 serine and 40 threonine residues. Over the years, numerous kinases have been reported to participate in the phosphorylation of Dlg and induce functional and positional changes in it (see figure 3). Earlier studies on Dlg phosphorylation concerned its role in signaling and localisation at the neuronal synapses. The first reported kinase was the p56lck tyrosine kinase which targets the amino terminus of Dlg

and causes coupling of tyrosine kinase and the voltage-gated potassium channel in T lymphocytes (Hanada et al., 1997). The CamKII kinase was shown to regulate Dlg localisation at the synapse (Koh et al., 1999). The PAR-1 kinase controls postsynaptic targeting of Dlg to neuromuscular junctions (Zhang et al., 2007). Recently, the importance of the phosphorylation of Dlg in a non-neuronal background has come to notice. Phosphorylation of hDlg on serine and threonine residues prevents its interaction with the p85 Src homology domain 2 in subconfluent intestinal epithelial cells, whereas phosphorylation of hDlg on tyrosine residues is essential for the same process (LaPrise et al., 2004).

The N terminus and PDZ regions hold a number of consensus serine and threonine sites for the MAPKs. The jun N-terminal Kinase (JNK) phosphorylates Dlg in response to osmotic stress and leads to its accumulation at cell-cell contacts (Massimi et al., 2006), and the p38γ MAPK dissociates Dlg from GKAP and consequently triggers its release from the cytoskeleton (Sabio et al., 2005). The hyperphosphorylation of Dlg has also been linked to its stability and turnover through interaction with the β-TrCP ubiquitin ligase (Mantovani and Banks, 2003), as well as to the formation of cell-cell contacts (Mantovani et al., 2001). The phosphorylation of the protein under osmotic shock has also been shown to have an effect on the interaction and subsequent degradation by the high-risk HPV E6 oncoprotein (Massimi et al., 2006). Although these above mentioned studies highlight the importance of Dlg phosphorylation in its spatial regulation, there have not been many reports on how it may affect the growth governing function of the protein with respect to its change in localisation. The PDZ binding kinase (PBK) (Gaudet et al., 2002) is the only kinase that has so far been potentially connected to a cell cycle regulating phosphorylation of Dlg. The PBK is a 322 amino acid serine/threonine kinase that was bound to the PDZ2 domain of Dlg through its C-terminal PBM in a yeast two hybrid assay. Both the kinase as well as Dlg was shown to be phosphorylated at mitosis in

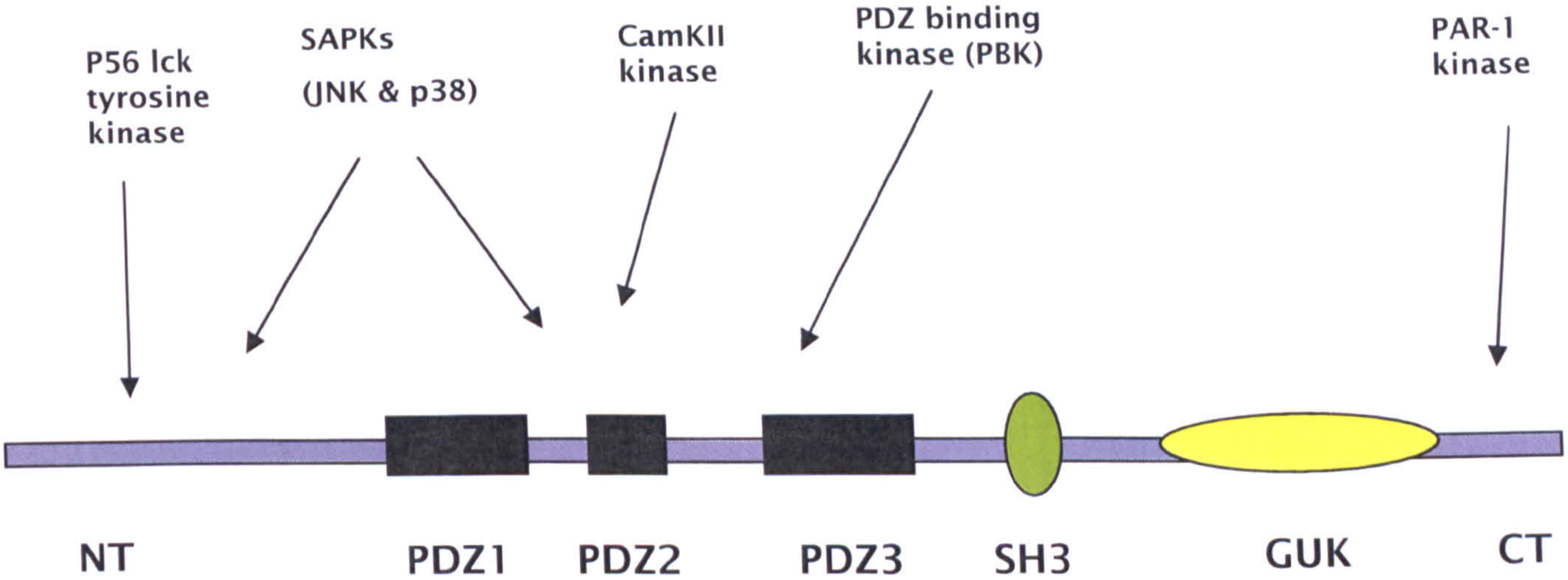


Figure 3: The phosphorylation of Dlg by various kinases.

A schematic of the Discs Large Protein that highlights its various domains and shows the kinases to which Dlg is a known substrate so far. The arrows point to the regions on the protein to which the kinases bind and cause phosphorylation.

HeLa cells, but although it is established that PBK is phosphorylated by the cyclin B- cdc2 complex, the claim that PBK mediates the mitotic phosphorylation event on Dlg remains to be verified. In addition, there is no other direct evidence that Dlg is a substrate for regulatory cell cycle kinases, nor is there any data on probable biological consequences of its phosphorylation during the cell cycle - information which will be fundamental in explaining the molecular and biochemical mechanisms behind the growth regulatory function of this tumour suppressor.

Thesis Aim

From the above introduction, it is clear that the Discs Large Tumour Suppressor is a multi-faceted protein that has an important role to play in a variety of cell types over diverse organisms, from flies to humans. It is also evident that as a protein it functions in different processes in the cell, such as in establishing polarity, in signaling, establishment of asymmetric division in Drosophila, in aiding organismal development and most importantly in controlling cellular growth and proliferation. The interaction of Dlg with its various cellular as well as viral protein partners seem to determine in a large way how it goes about fulfilling all these different tasks in the cell.

Many recent studies (Zhang et al., 2007; Massimi et al., 2006; Sabio et al., 2005; LaPrise et al., 2004; Mantovani and Banks 2003) have highlighted the importance of the presence or absence of a phosphorylated form of Dlg to certain cellular processes, and how it significantly affects the localisation and function of the protein. Given the involvement of Dlg in the control of cellular proliferation and signaling events, and the previously recognized role of phosphorylation in proteins that have such functions, deciphering and understanding the role of this modification of Dlg as a means to comprehend the function of this protein assumes a great amount of value.

The aim of this thesis is to examine the effect of various phosphorylation events on the function and localisation of hDlg.

Results

Part 1: The Phosphorylation of Dlg Under Osmotic Stress

Osmosensing or the registration of cell volume, triggers signal transduction pathways towards effector sites which link alterations of cell volume to a functional outcome. Inducing osmotic stress on cells using sorbitol is a method exploited to study the subsequent activation of the SAPK/MAPK pathway which includes the erk1/2, the JNK kinase, and the p38 MAPK - which is the mammalian homologue of the Yeast protein Hog1 which is involved in a similar response in Yeast (Haussinger et al., 1999; Kayali et al., 2000; Diker-Cohen et al., 2003). The mammalian cell has been shown to sense osmotic shock by using the mitochondria and its outer membrane associated protein mTor (Desai et al., 2002) as well as the cation channel protein OTRPC4 as molecular sensors, and has been shown to affect several signal transduction pathways including those involved in regulation of metabolism, gene expression and the susceptibility to stress (Schliess et al., 2007). In response to osmostress, cells transiently modulate cell-cycle progression to allow adaptation. For instance, to protect cells from hypertonicity, p53 inhibits DNA replication and transition from G_1 to S (Dmitrieva *et al.*, 2001), and p53 is also known to be phosphorylated in response to stress by p38 MAPK. G_2-S and M delays seem to be p53-independent. Hypertonicity causes a rapid activation of the G_2-M checkpoint through activation of p38, which causes a drop in Cdc2 kinase activity (de Nadal et al., 2007).

Given that the system of inducing osmotic shock has already been used to study the activation of these kinase pathways as a consequent means to study phosphorylation of important players such as p53 by those pathways (Kishi et al., 2001), we chose to use the system to investigate hDlg phosphorylation. Though

induction of osmotic shock is a standard procedure for monitoring the effects of the MAPK signal transduction cascade on the regulation of a given protein complex, in the case of keratinocytes this has the added relevance of representing one of the normal stress conditions to which the cells are exposed; it can induce hyper-polarization (Gonczi et al., 2007), and recent data also suggests that osmotic shock can induce the differentiation programme in keratinocytes (Mammone et al., 2008), which in turn is also regulated by the various components of the MAPK pathway (Eckert et al., 2002).

Since hDlg has multiple potential sites of phosphorylation by MAPKs (Figure 4), a number of which have subsequently been confirmed, we chose the use of sorbitol as a means of investigating two events: The effect of osmotic stress on the pattern of hDlg expression, and whether this behaviour correlated with the activation of the above-mentioned kinase pathways and subsequent hDlg phosphorylation.

Accumulation of hDlg in mammalian cells at sites of cell-cell contact following osmotic stress

To examine the effect of osmotic stress on the localisation of hDlg, HaCaT cells, which are a human skin epithelial line, and CaCO2, which are an intestinal epithelial line, were grown on glass coverslips and subjected to 0.3M sorbitol for one hour. Cells were then fixed and the pattern of hDlg expression analysed by immunofluorescence. hDlg exhibited an accumulation at sites of cell-cell contact following osmotic stress in both cell lines as shown in Figure 5. Since hScrib and Hugl-1 form part of the same polarity complex, it was of obvious interest to investigate their pattern of expression under similar stress conditions. To do this, HaCaT epithelial cells grown on coverslips were exposed to 0.3M sorbitol for 1hr, and the pattern of hDlg, hScrib and Hugl-1 expression was determined by

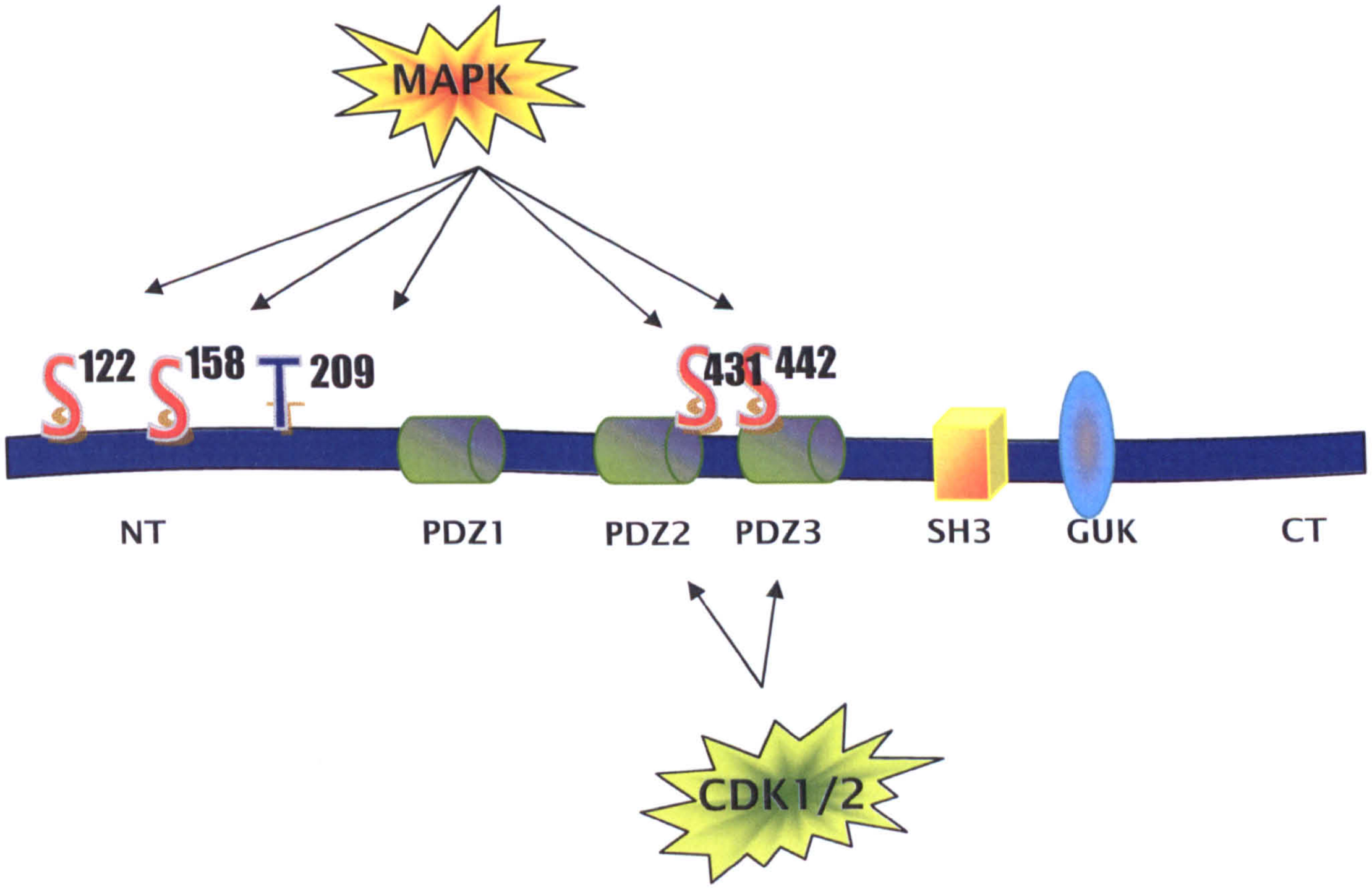


Figure 4: A schematic representation of the hDlg protein with major domains highlighted. The phosphorylation of the highlighted residues are described in the following pages.

Figure 5a

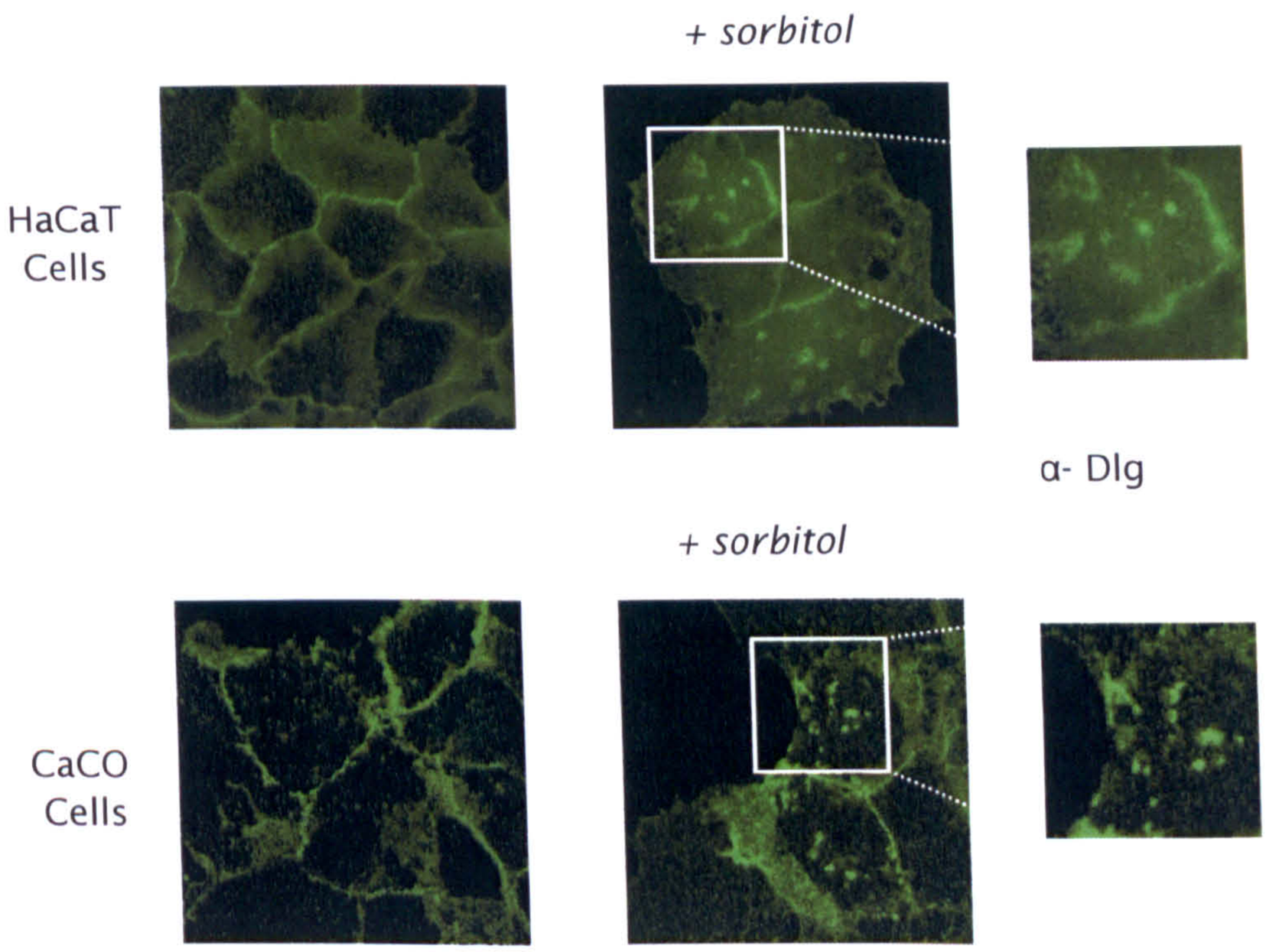


Figure 5: Accumulation of the hScrib Polarity complex at sites of cell contact following osmotic stress

a) HaCaT and CaCo epithelial cells were plated and grown on glass coverslips overnight and then exposed to 0.3M sorbitol for 1h. The cells were then fixed and stained with the Dlg 2d11 monoclonal antibody.

immunofluorescence analysis. Specific antibodies were used in pair wise combinations to directly compare the respective patterns of expression of the three proteins, and the results obtained are shown in Figure 6a and Figure 6b. As can be seen, there is a clear accumulation of the hDlg protein at sites of cell contact following osmotic stress. In addition, a very similar pattern of increased membrane staining is observed for both hScrib and Hugl-1. It is also apparent that there is a significant degree of colocalisation between the three proteins in untreated cells, but this increases significantly following exposure to osmotic stress. Finally, it is noteworthy that hDlg, hScrib and Hugl-1 also show accumulation in vesicular-type structures within the cytoplasm following osmotic shock (see insets to figure 6), where, once again, there is a very significant degree of colocalisation. These results demonstrate that hDlg, hScrib and Hugl-1 all exhibit increased levels of expression at sites of cell-cell contact following exposure to osmotic stress.

Although the increased co-localisation of the polarity complex implies redistribution of hDlg to the cell junctions, an alternative possibility might also be that sorbitol induced osmotic stress induces proteolytic degradation of the cytoplasmic pools of the protein and stabilization of the membranous forms, thereby giving a misleading impression of relocalisation. To investigate this further, we performed a subcellular biochemical fractionation assay of HaCaT cells that were untreated, exposed to osmotic shock and exposed to osmotic shock in the presence of proteasome inhibitors. The fractionation assay, performed using the Calbiochem ProteoExtract Subcellular Protein Extraction Kit yields four different fractions from cells - Fraction 1 (F1) which is the cytosol, Fraction 2 (F2) which consists of the organelles and the membrane, Fraction 3 (F3) is the nuclear fraction, while Fraction 4 (F4) consists of the cytoskeleton. The pattern of hDlg expression was then analysed by western blotting and Lamin A/C - which is found specifically in the nucleus and the nuclear membrane - and α-tubulin - which is a specific component of the cytosol and cell membrane - were used as markers to verify the integrity of

the extraction procedure. The results obtained are shown in Figure 7. As can be seen, there is an increase of Dlg in the cytoskeletal fraction upon induction of osmotic stress, and this is accompanied by a corresponding decrease in the membrane and nuclear pools of the protein. The presence of the proteasome inhibitor MG-132, does not affect the levels of Dlg expression in the fractions differentially, and shows a moderate increase in all pools proving that the specific increase in the cytoskeletal fraction seen on osmotic shock is not a by-product of proteasome degradation or protein turnover (Figures 7a and 7b). These results further support the notion that hDlg actively changes location within the cell following exposure to osmotic stress.

hDlg is phosphorylated under osmotic stress

JNK phosphorylation of hDlg under osmotic stress leads to its relocalisation

It is known that sorbitol induced osmotic stress causes phosphorylation and activation of certain kinases in the MAPK/SAPK pathway including JNK, erk1/2 and p38 (Kavali et al., 2000). We were interested in investigating whether any of these kinases might play a role in the relocalisation of Dlg. To do this, we used HaCaT cells grown on coverslips and then subjected them to treatment with different kinase inhibitors - the ERK1/2 inhibitor (PD98059) was used at a concentration of 10μM, the p38alpha/beta inhibitor (SB203580) was used at a concentration of 20μM and the JNK inhibitor (SP600125) was used at a concentration of 10μM. The cells were first pre-treated for half an hour with the inhibitor, after which sorbitol coupled with the inhibitor was added to them for a further hour. The cells were then fixed in 3.7% Para formaldehyde and subjected to immunofluorescence. The untreated cells, with and without sorbitol, as shown in Figure 8a, show the expected pattern of hDlg localisation, with the sorbitol treated ones showing enrichment at the junctions and appearance of vesicular structures. Among the cells treated with the kinase

Figure 6a

Dlg / Scribble

+ sorbitol

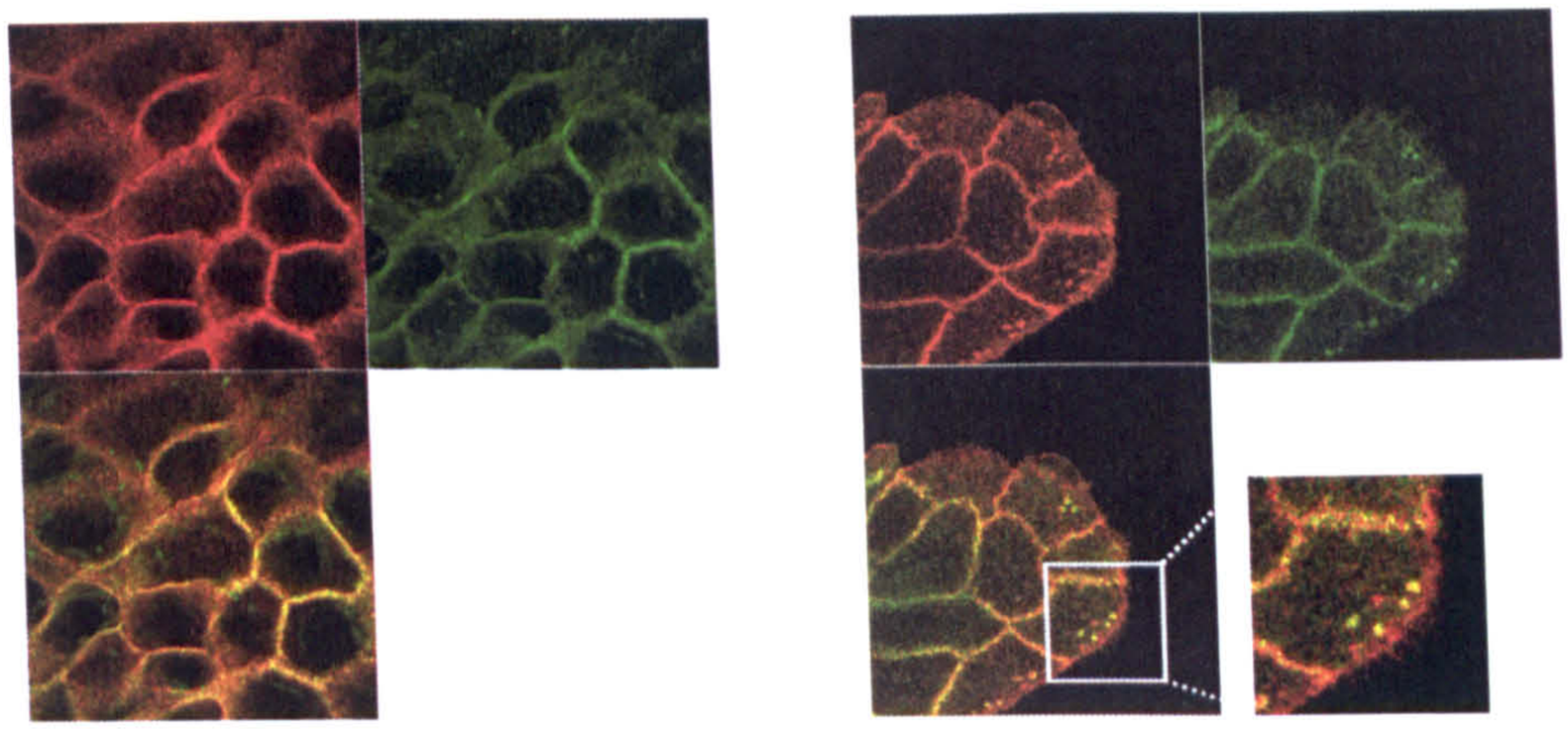


Figure 6b

Dlg / Hugl-1

+ sorbitol

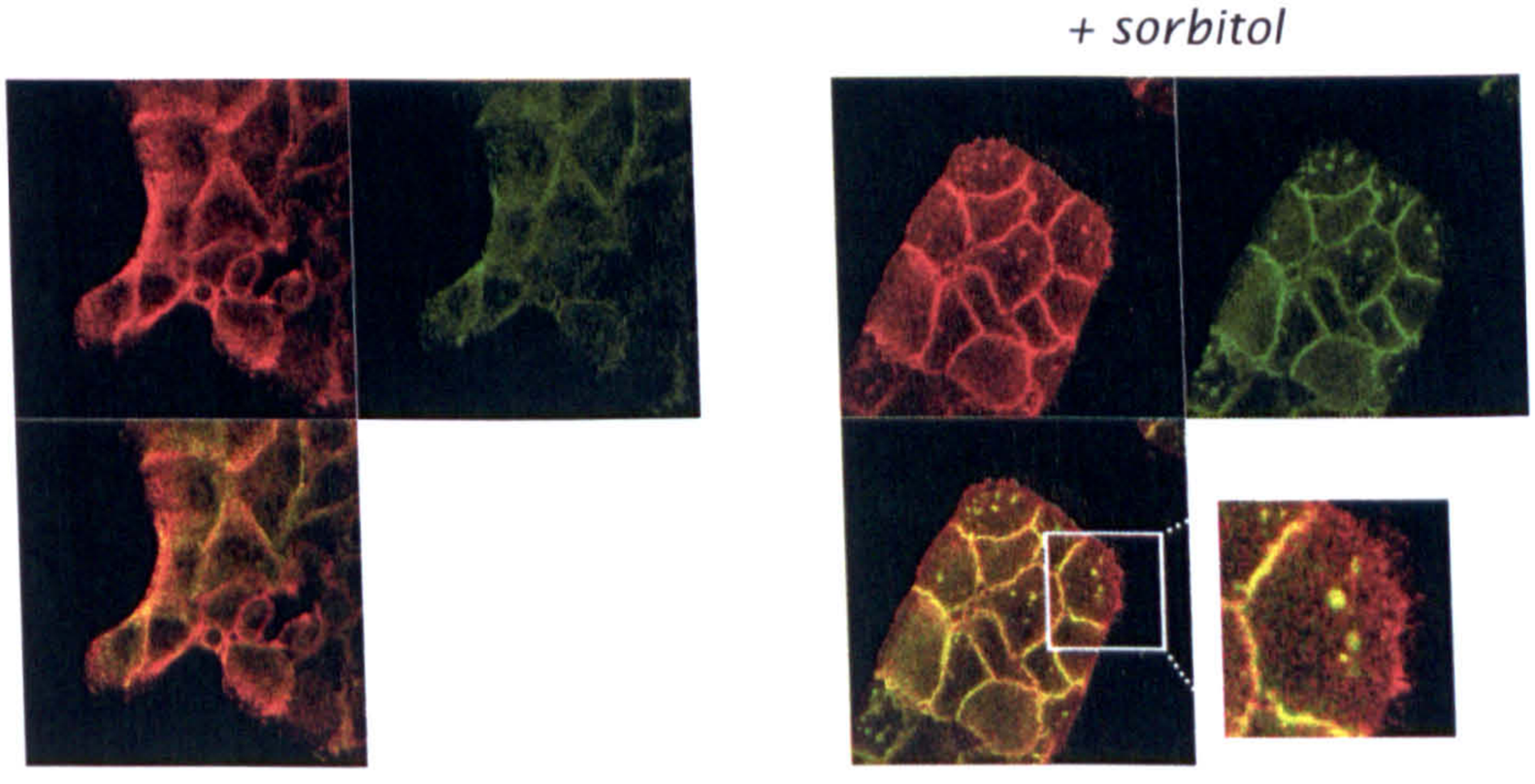


Figure 6: Accumulation of the hScrib Polarity complex at sites of cell contact following osmotic stress

HaCaT cells were treated as before and stained in pairwise combinations for a) Dlg (green) and Scribble (red) and b) Dlg (red) and Hugl-1 (green).

Figure 7a

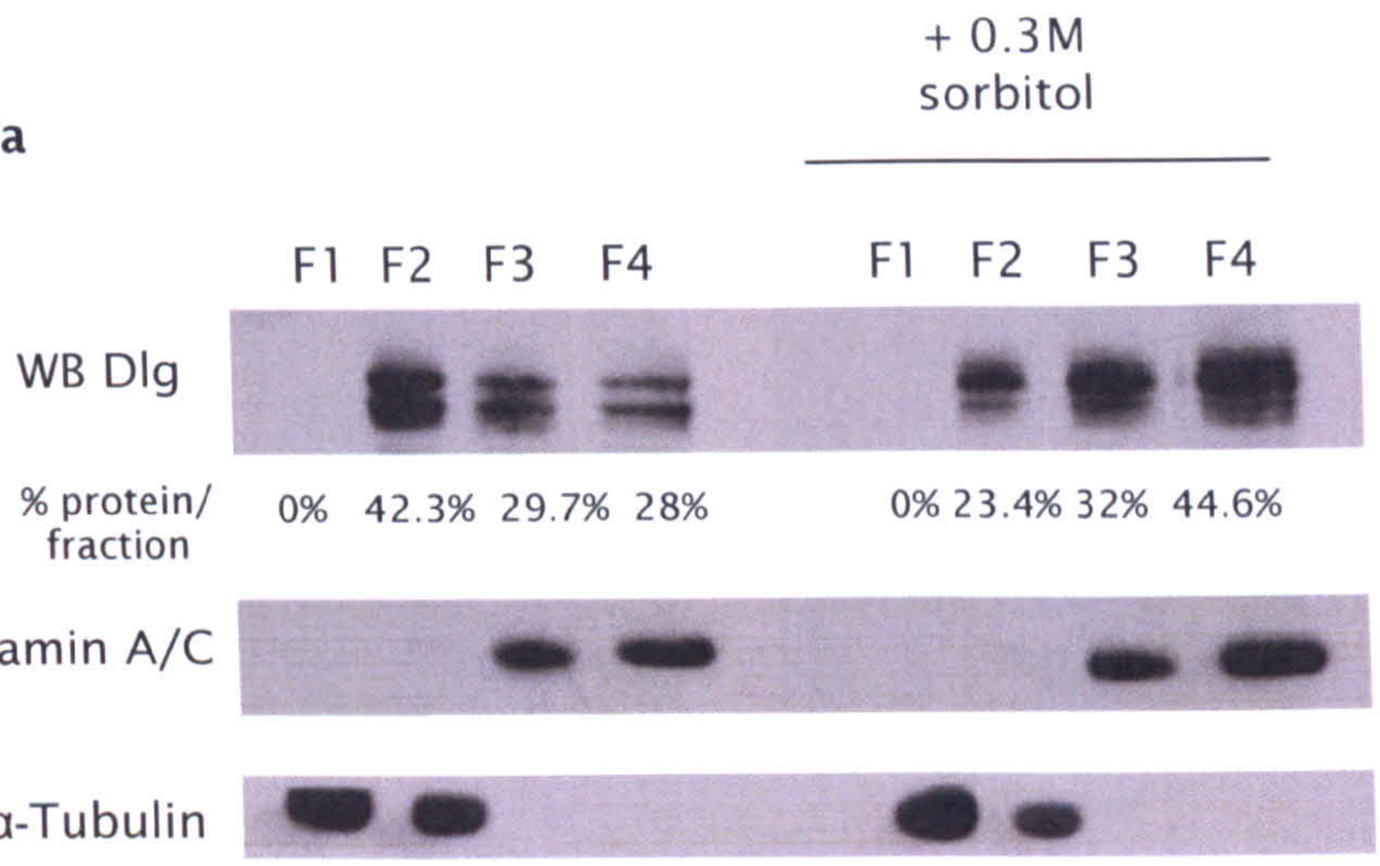
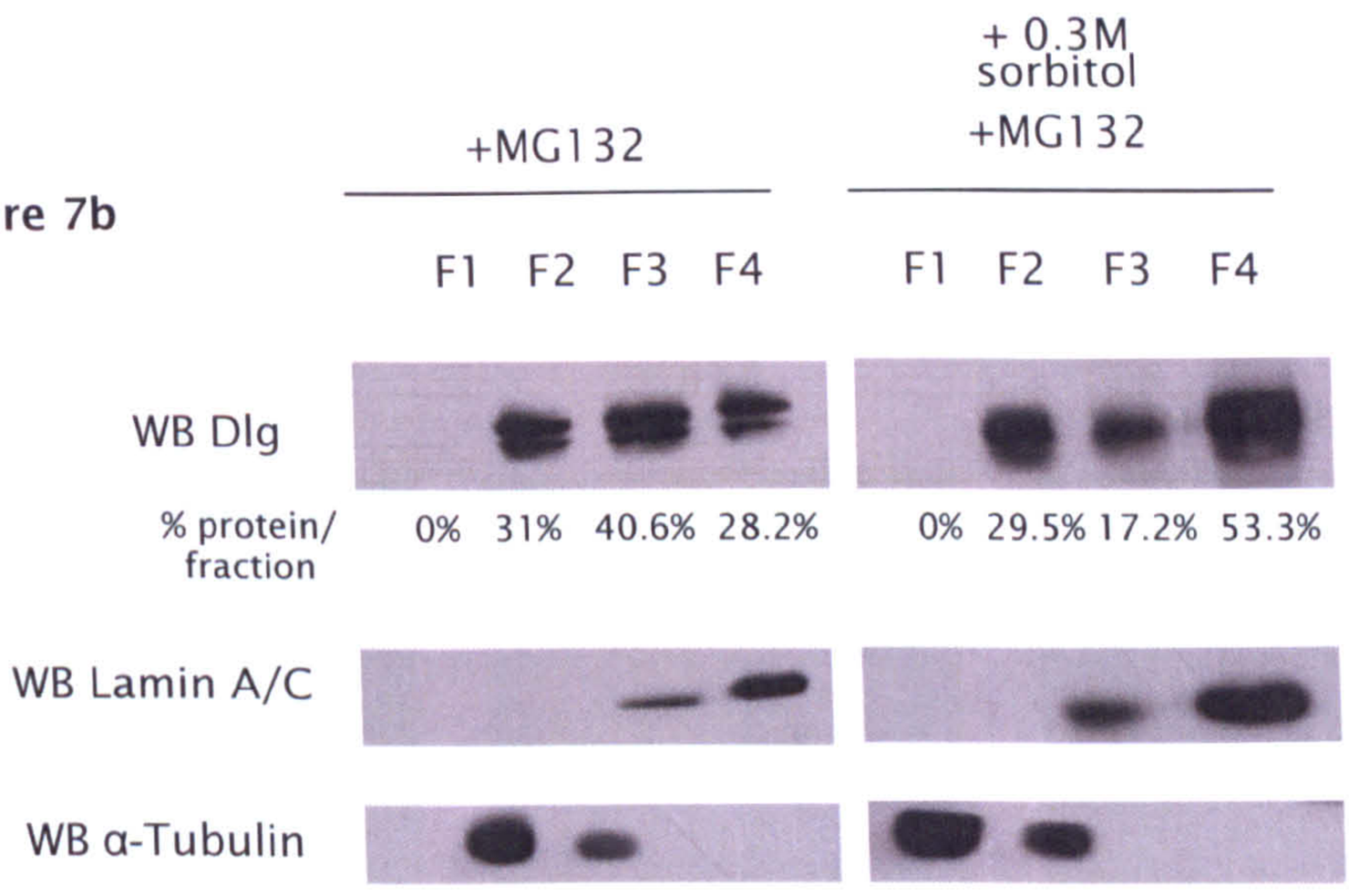


Figure 7b



F1 – Cytosolic fraction

F2 – Organelle/Membrane fraction

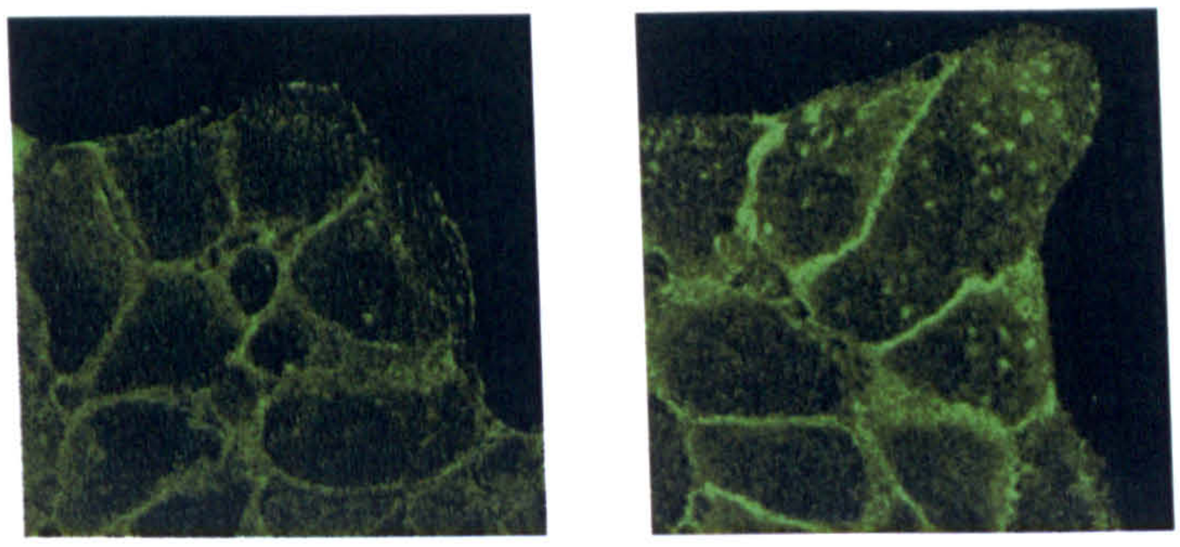
F3 – Nuclear fraction

F4 – Cytoskeletal fraction

Figure 7: Osmotic shock induced relocalisation of hDlg

Relocalisation of hDlg to an insoluble cytoskeletal fraction following osmotic stress is demonstrated by differential extraction of HaCaT cells. HaCaT cells were grown for 24hrs, then exposed to a) 0.3M sorbitol for 1hr, and b) the proteasome inhibitor MG-132 alone and 0.3M sorbitol plus MG132. Cells were then differentially extracted using the Calbiochem ProteoExtract Subcellular Protein Extraction Kit, and protein expression ascertained by western blotting. The percentage of protein per fraction was quantified using densitometry. Lamin A/C and α-Tubulin are used as markers for the fractions shown.

Figure 8a



+ sorbitol

Figure 8b

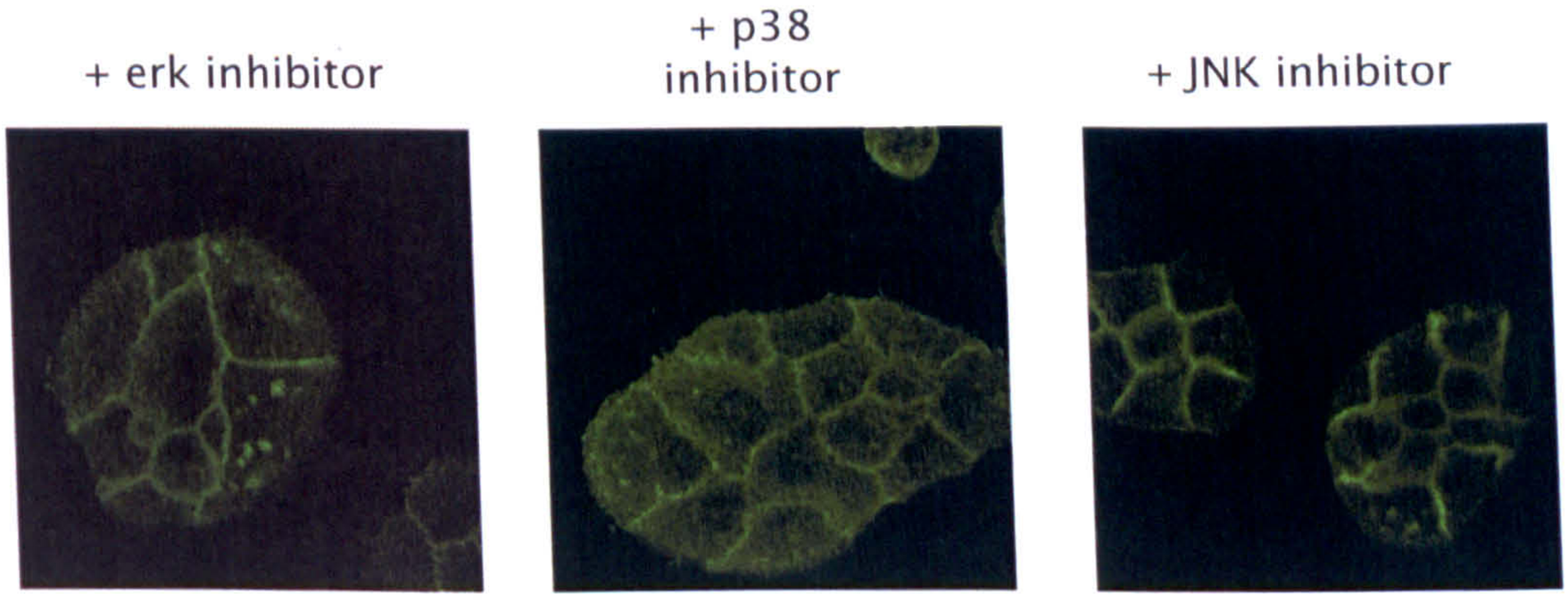


Figure 8: Relocalisation of hDlg following osmotic shock is dependent on its phosphorylation by JNK

a) HaCaT cells stained for hDlg before and after sorbitol treatment

b) And c) HaCaT cells were treated with various inhibitors - the erk inhibitor, the p38 inhibitor and the JNK inhibitor for 3 hours post sorbitol treatment. Note that there is no appearance of vesicular structures in the panel treated with the JNK inhibitor or enrichment of the protein at junctions.

inhibitors (Figure 8b), treatment with p38 and ERK1/2 inhibitors had minimal effect upon the formation of the vesicular structures containing hDlg. In contrast, treatment with JNK inhibitors blocks the formation of the vesicular structures. A similar pattern is seen also with hScrib (figure 9a and 9b), where the appearance of the vesicular structures is prevented on the addition of the JNK inhibitor. However all three inhibitors seem to marginally affect the targeting of hScrib and hDlg to the junctions. These results demonstrate that the osmotic shock-induced phosphorylation of Dlg by JNK targets Dlg to vesicular structures in the cell and that hScrib, another member of the polarity complex which includes hDlg, is also affected by it.

hDlg localises to endosomes upon osmotic shock

Next, we sought to examine if the numerous vesicular structures to which hDlg localises to upon osmotic shock, might be endosomal structures. Endosomes are membrane bound transport organelles within cells which function in the trafficking and recycling of several different types of cellular components including proteins, to and from the cell membrane (Wall and Maack, 1985). It seemed probable that hDlg and other partnering members of the hScrib polarity complex, upon phosphorylation might be carried on these structures to reach the plasma membrane, explaining the enrichment of these proteins in that part of the cell upon osmotic shock. To test this hypothesis, we used two approaches.

First, we wanted to look at the localisation of the structures themselves with respect to hDlg. To do so, HaCaT cells were grown on coverslips, exposed to sorbitol for one hour and subjected to staining with specific antibodies to hDlg and Transferrin, which is a well known marker for early endosomes in cells (Dautry-Varsat, 1986). It must be noted that Transferrin is also found in recycling endosomes and the colocalisation seen between the two proteins might also be a

result of the recycling of Dlg to and fro from the membrane. From figure 10a, it is clear that there is very little colocalisation between the two proteins under normal circumstances. However, on inducing osmotic shock (figure 10b), there is a dramatic appearance of dot-like structures and increased staining at the membrane in which both proteins colocalise to a large degree. This therefore indicates that at least some of the structures into which hDlg localises upon osmotic shock are early endosomes. It is worth noting that often a very small number of these structures are also seen in untreated cells, suggesting that in non stressed conditions, this might be one of the ways in which hDlg is transported to the cellular membrane.

To further support the notion that these structures are indeed endosomes we analysed the pattern of hDlg expression in the presence of an inhibitor of endosomal transport. The inhibitor used was 5μM Sodium Monensin, which is a potent blocker of vesicular transport in eukaryotic cells (Uchida et al., 1979). HaCaT cells were cultured on coverslips overnight, and were then treated with 5μM Sodium Monensin alone (figure 11b), or with sorbitol (figure 11c) or pre-treated with 5μM Sodium Monensin for 30 minutes and then the culture medium was replaced with medium containing both 0.3M sorbitol and sodium monensin (figure 11d) for a further hour. As is visible from Figure 11d, the sorbitol induced accumulation of hDlg in vesicular structures and its enrichment at the junctions is reduced upon the treatment with sodium monensin. These results indicate that hDlg trafficking at least in part depends on the localisation of the protein into endosomal structures.

p38 MAPK phosphorylation of hDlg under osmotic stress makes it more susceptible to degradation by HPV E6

Having found that phosphorylation of hDlg can induce major changes in the pattern of hDlg localisation, the next question to be addressed was whether these

Figure 9a

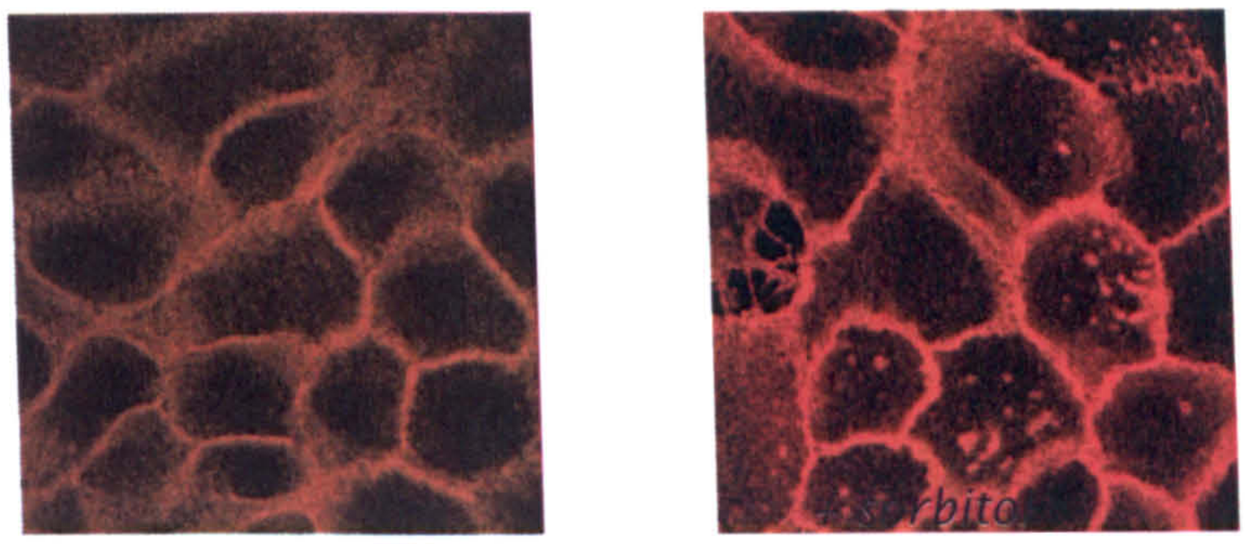


Figure 9b

Figure 9: Relocalisation of hScrib following osmotic shock is dependent on its phosphorylation by JNK

a) HaCaT cells stained for hScrib before and after sorbitol treatment

b) And c) HaCaT cells were treated with various inhibitors - the erk inhibitor, the p38 inhibitor and the JNK inhibitor for 3 hours post sorbitol treatment. Note that there is no appearance of vesicular structures in the panel treated with the JNK inhibitor or enrichment of the protein at junctions.

Figure 10a

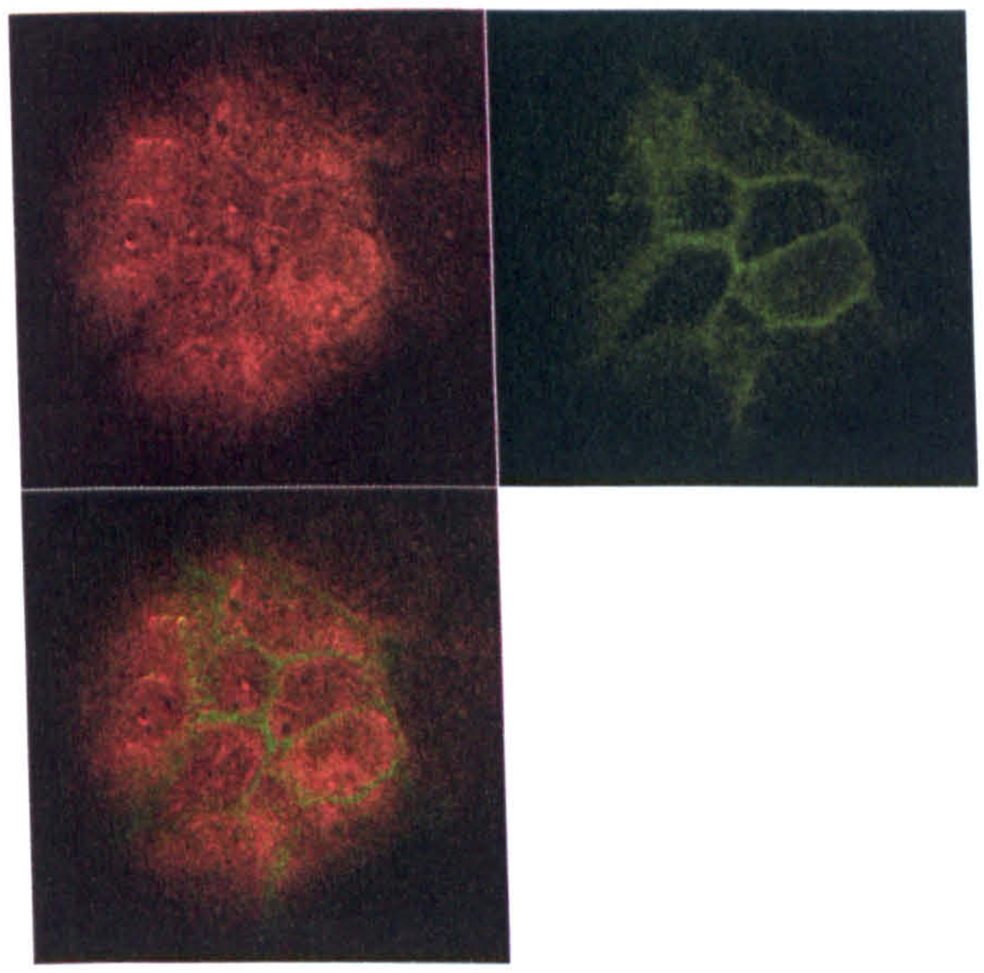
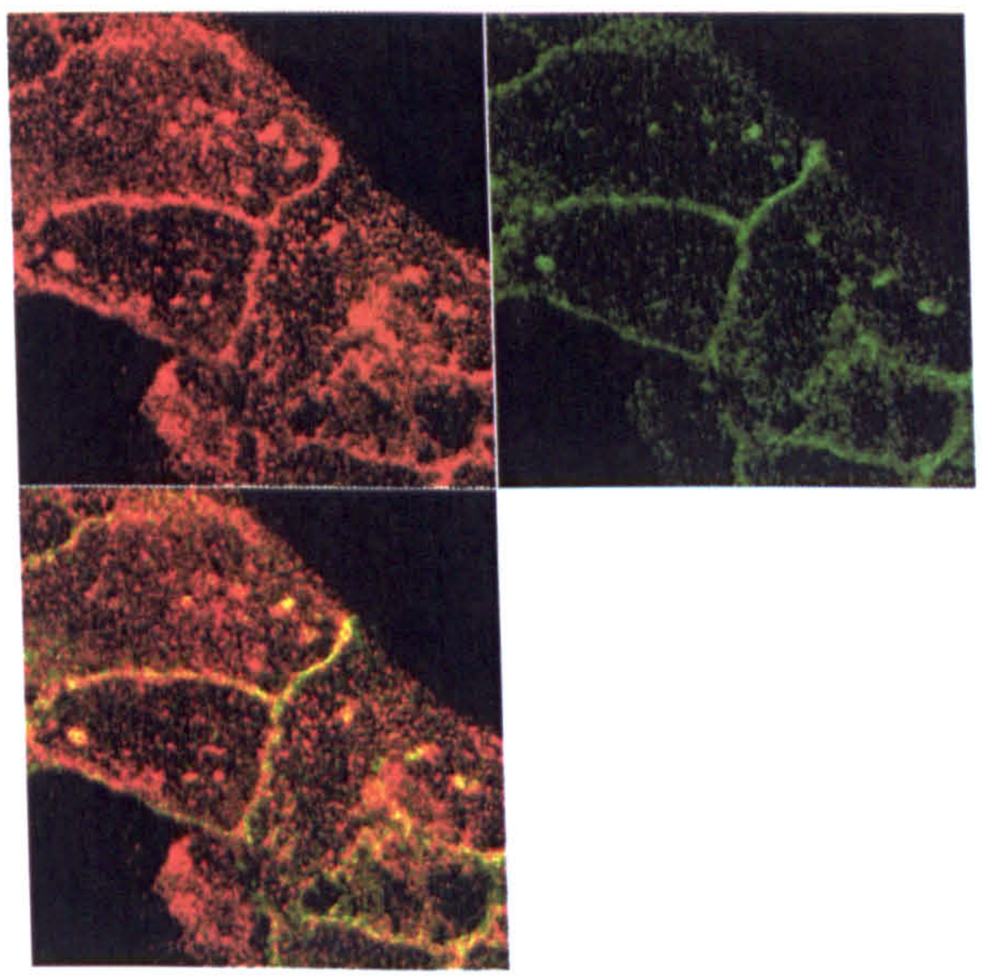


Figure 10b



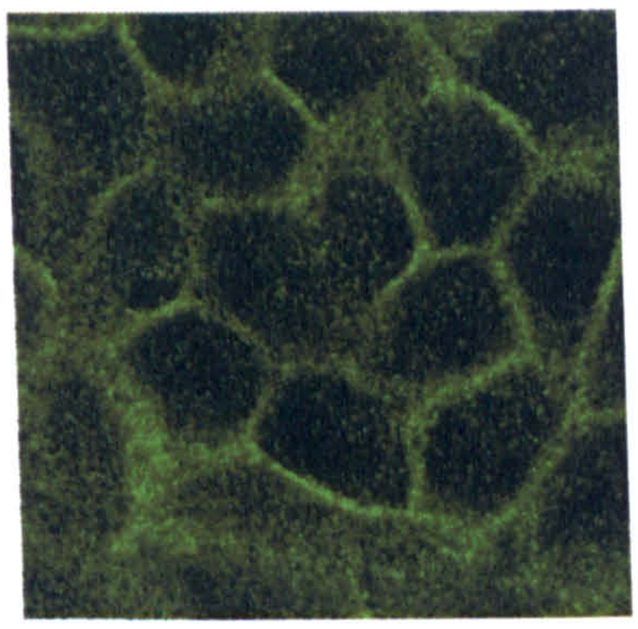
+ sorbitol

Dlg/Transferrin

Figure 10: Colocalisation of Dlg with the Early Endosomal Marker Transferrin upon Osmotic Shock.

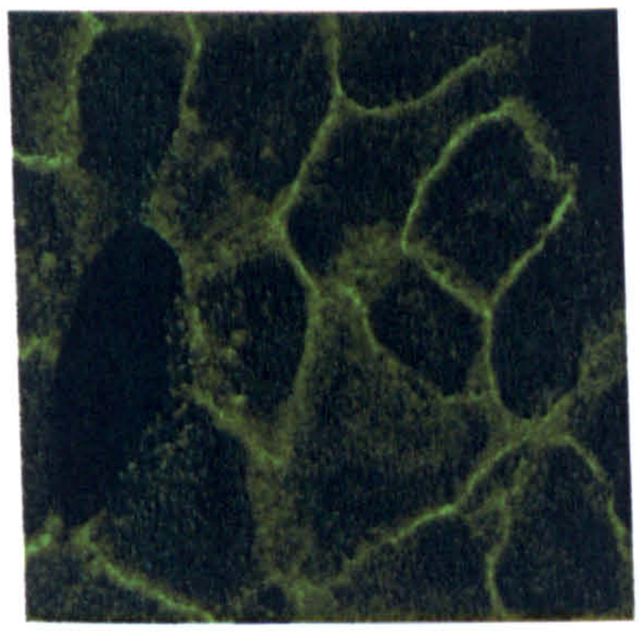
a) without and b) with sorbitol treatment. HaCaT cells were plated on coverslips and then left untreated (panel a) or treated with sorbitol for 1h (panel b). The cells were then fixed and stained in combination for hDlg (green) and the early endosomal marker transferrin (red).

Figure 11a



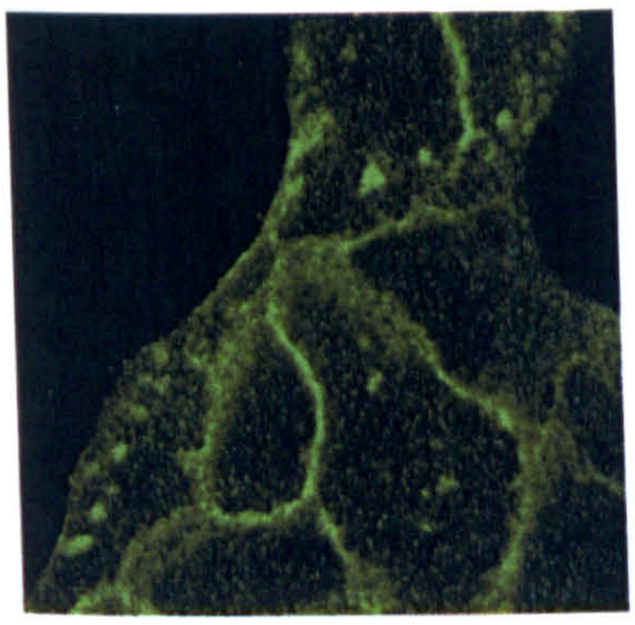
Untreated

Figure 11b



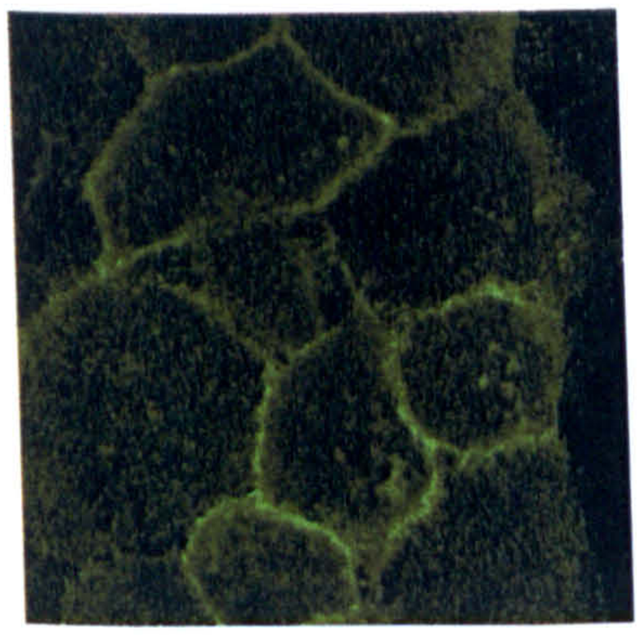
+ Na Monensin

Figure 11c



+ sorbitol

Figure 11d



Na Monensin
+
Sorbitol

Figure 11: Analysis of hDlg expression in cells treated with the endosomal inhibitor Sodium Monensin

HaCaT cells were cultured on coverslips and treated with b) sorbitol or c) pretreated for 30 minutes with sodium monensin, followed by one hour of both sorbitol and sodium monensin. The coverslips were then fixed and stained with the Dlg 2d11 monoclonal antibody

phosphorylation events may in any way alter the susceptibility of hDlg to HPV E6 induced degradation. Indeed, Mantovani et al (2001) showed that the proteasome mediated regulation of hDlg in cells to be dependent on its hyperphosphorylation and stabilisation. Therefore we decided to use the osmotic stress model as a way of inducing hyperphosphorylation of hDlg and thereby investigating the susceptibility of hDlg to HPV E6 induced degradation. In addition, recent studies have also shown that Dlg is subject to phosphorylation by the p38 MAPKs and JNK following osmotic shock and that these phosphorylation sites lie within the amino terminal half of the protein (Sabio *et al.*, 2005; Massimi *et al.*, 2006). Since we had already seen a significant shift in the migration pattern of hDlg expression (Figure 5), we wanted to investigate how much of this was due to phosphorylation. Human 293 cells were used for this experiment, given the technical difficulty in obtaining a decent transfection efficiency in HaCaT cells. 293 cells were transfected with Dlg and subjected to osmotic shock. Subsequently Dlg was immunoprecipitated using anti-HA antibody and then analysed by western blotting with an anti phospho-serine/threonine specific antibody. The results obtained in Figure 12a demonstrate that Dlg is weakly phosphorylated in untreated cells but that this increases significantly following treatment with sorbitol. This suggests that although there are residues on Dlg that are constitutively phosphorylated (Sabio et al., 2005); phosphorylation of other residues is enhanced under conditions of osmotic shock.

To then investigate the susceptibility of phosphorylated Dlg to E6-induced degradation, cells were transfected with Dlg plus increasing amounts of an HPV-18 E6 expression plasmid. After 24h the cells were exposed to sorbitol for 1h and residual Dlg was measured by western blot analysis. The results obtained are shown in Figure 12b where it can be seen that the slower migrating, hyper-phosphorylated form of Dlg, is significantly more susceptible to E6-induced degradation. To exclude the possibility that this might be due to an effect of the sorbitol on E6 activity, the

assay was repeated with the ΔNT mutant of Dlg which lacks the first 185 amino acids of the protein. As can be seen from Figure 12c there is no change in the efficiency with which E6 targets this mutant for degradation, irrespective of whether sorbitol is present or not. Sabio et al., identified five phospho-acceptor sites on Dlg which lie in the N terminus and the PDZ regions of the protein (Figure 13a), of which serine 122 was the only one reported to be constitutively phosphorylated. Therefore we wanted to determine which of the residues might be responsible for the enhanced targeting by HPV-18 E6. Since the N terminus seems to play a critical role, we focused on two potentially relevant mutants - Serine 158 and Threonine 209. The five residues were all individually mutated to alanines to render them resistant to phosphorylation on the respective residues and expressed in vivo as GST fusion proteins (a generous gift from Dr. Ana Cuenda). We first analysed the effects of osmotic shock upon the migration pattern of these mutants and the results in Figure 13b shows that each phospho-mutant, with the exception of Serine 158, show higher migrating phosphorylated forms of the protein upon treatment with sorbitol. This suggests that phosphorylation on serine 158 may be responsible for triggering a cascade that leads to subsequent hyper-phosphorylation on other residues (see discussion).

To investigate the susceptibility of N-terminally phosphorylated hDlg to HPV 18E6 further, we repeated the assay using two mutants of Dlg at residue S158, which lies within this region and S209, which lies just outside. Cells were transfected with the wild type and mutant Dlg expression plasmids, together with increasing amounts of E6 expression plasmid. Following exposure to osmotic shock cells were harvested and the remaining Dlg was assessed by western blot analysis using anti GST antibodies. The results obtained are shown in Figure 14 and demonstrate that wild type (figure 14a) and the S209 (figure 14c) mutant of Dlg are both subject to increased levels of E6-induced degradation following exposure to osmotic shock. In

Figure 12a

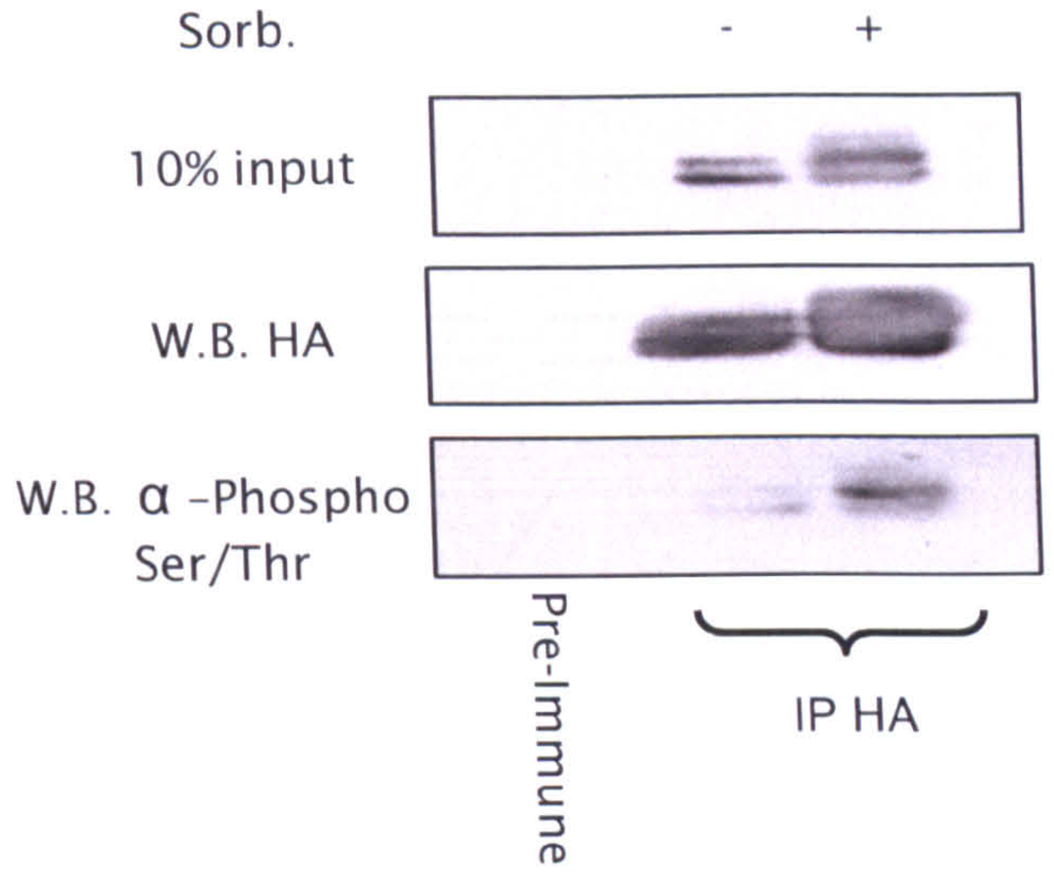


Figure 12b

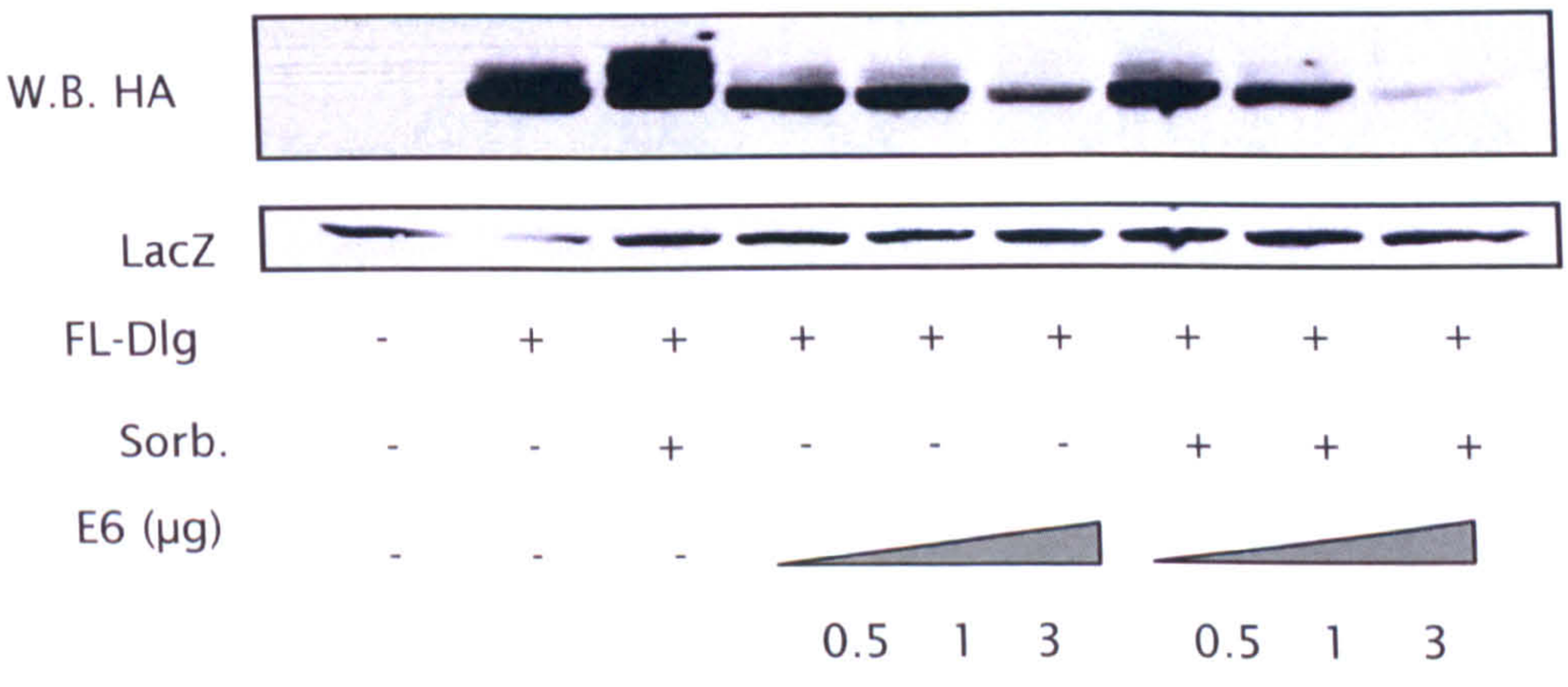
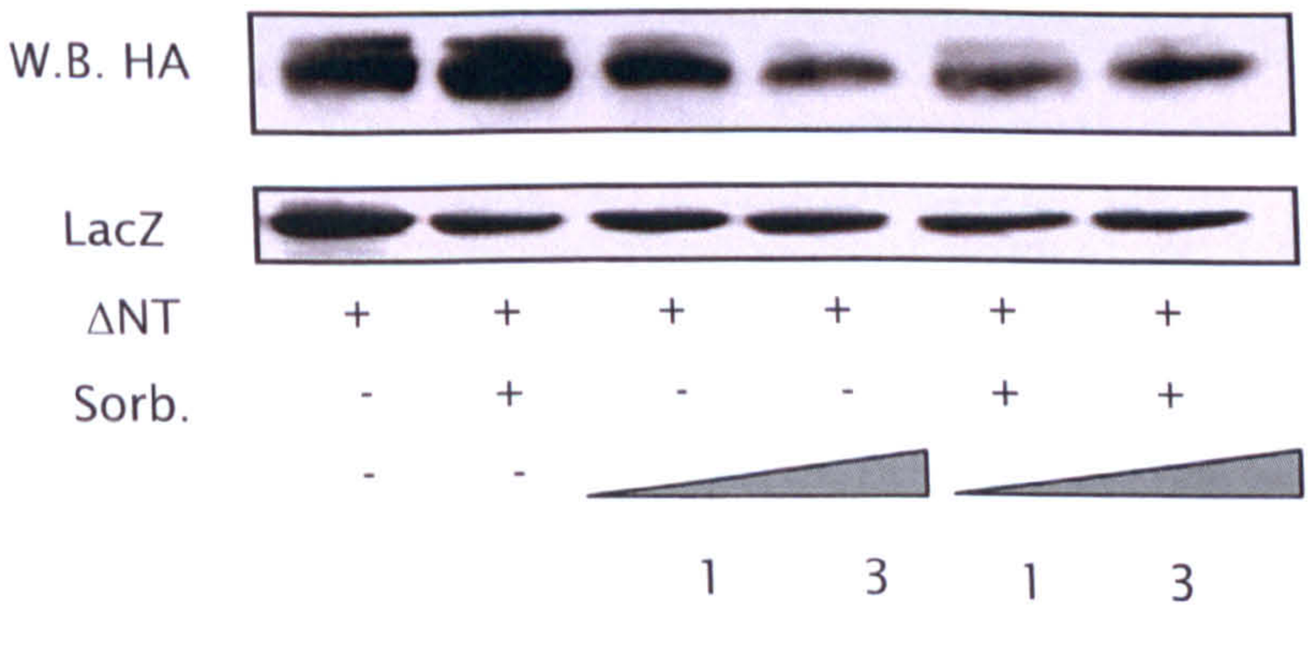


Figure 12c



Courtesy L.Banks

Figure 12: Dlg is phosphorylated under osmotic shock and phosphorylation on its N terminus makes it more susceptible to HPV 18 E6 induced degradation

a) HEK 293 cells transfected with HA-Dlg were either incubated with or without sorbitol for one hour, after which the cells were lysed in E1A buffer, immunoprecipitated onto HA-agarose beads and subjected to western blotting. The blot was then probed with an anti-HA antibody to detect the transfected protein as well as with an anti phospho-Serine/Threonine antibody to detect phosphorylation. HEK 293 cells transfected with either b) FL (Full length)-Dlg or c) a construct of Dlg lacking the N terminus, ΔNT, were either incubated with or without sorbitol for one hour with increasing amounts of HPV 18E6. The cells were then lysed with E1A buffer and subjected to western blotting, after which the blot was probed with an anti-HA antibody to detect the transfected protein and LacZ as a transfection control.

Figure 13a

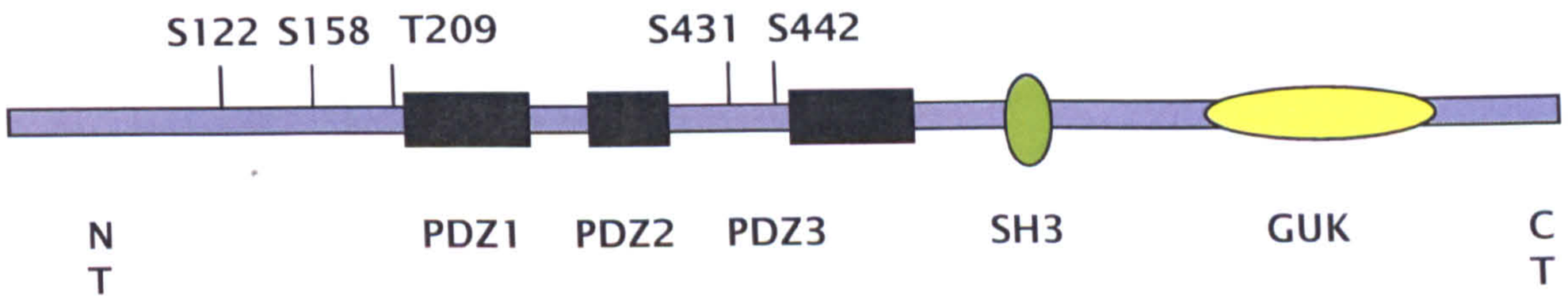


Figure 13b

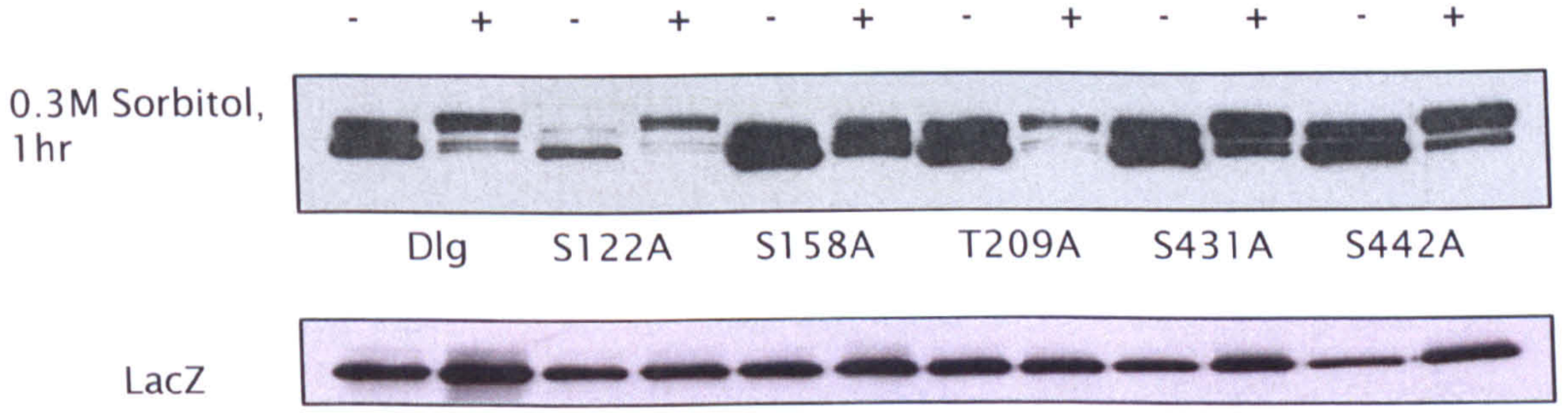


Figure 13: Phosphorylation of hDlg by the p38MAPK in response to osmotic shock

a) Illustration showing the position of the five residues on hDlg which are phosphorylated by the p38 MAPK in response to osmotic shock. b) HEK 293 cells transfected with the Dlg phospho-point-mutants for 24 hrs and treated with 0.3M sorbitol for one hour. The cells were then lysed and subjected to western blotting. The blot was probed with an anti-GST antibody to detect Dlg, and LacZ was usedas a transfection control.

Figure 14a

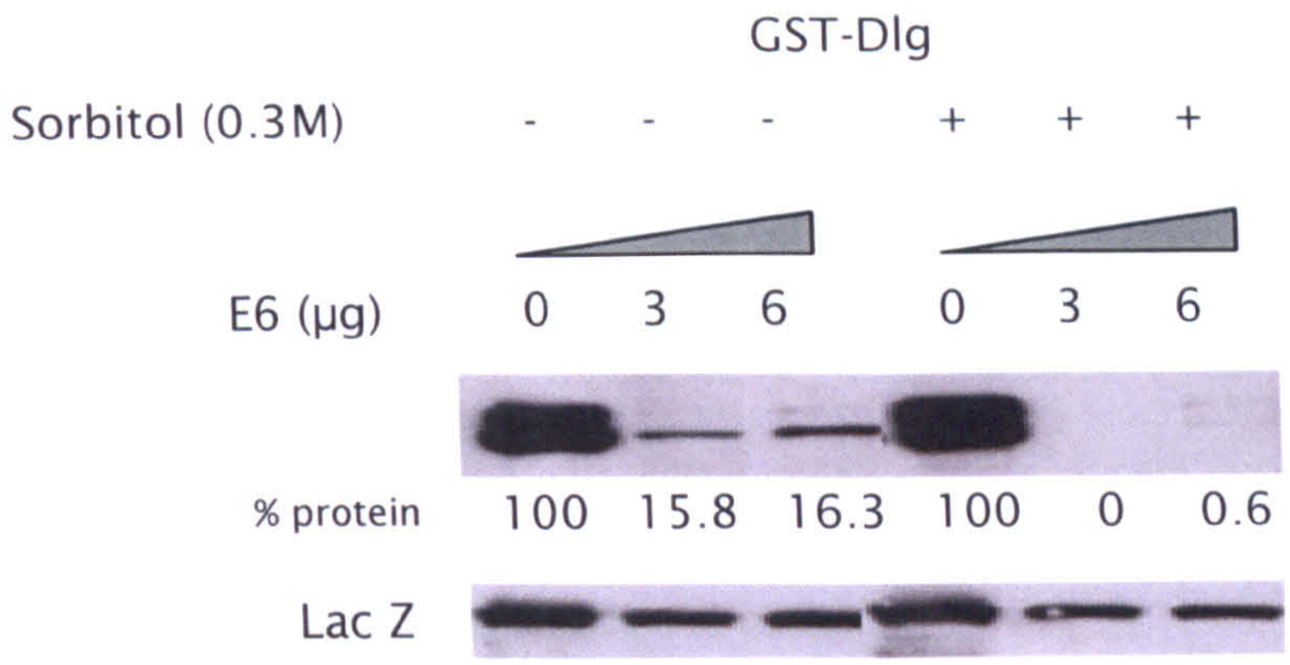


Figure 14b

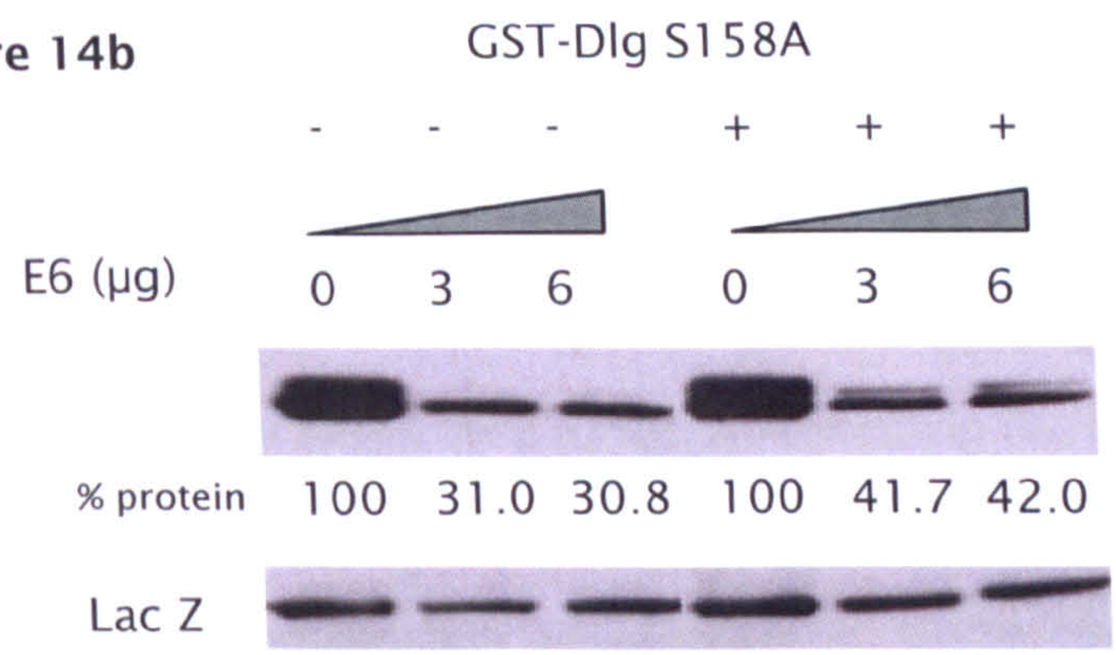


Figure 14c

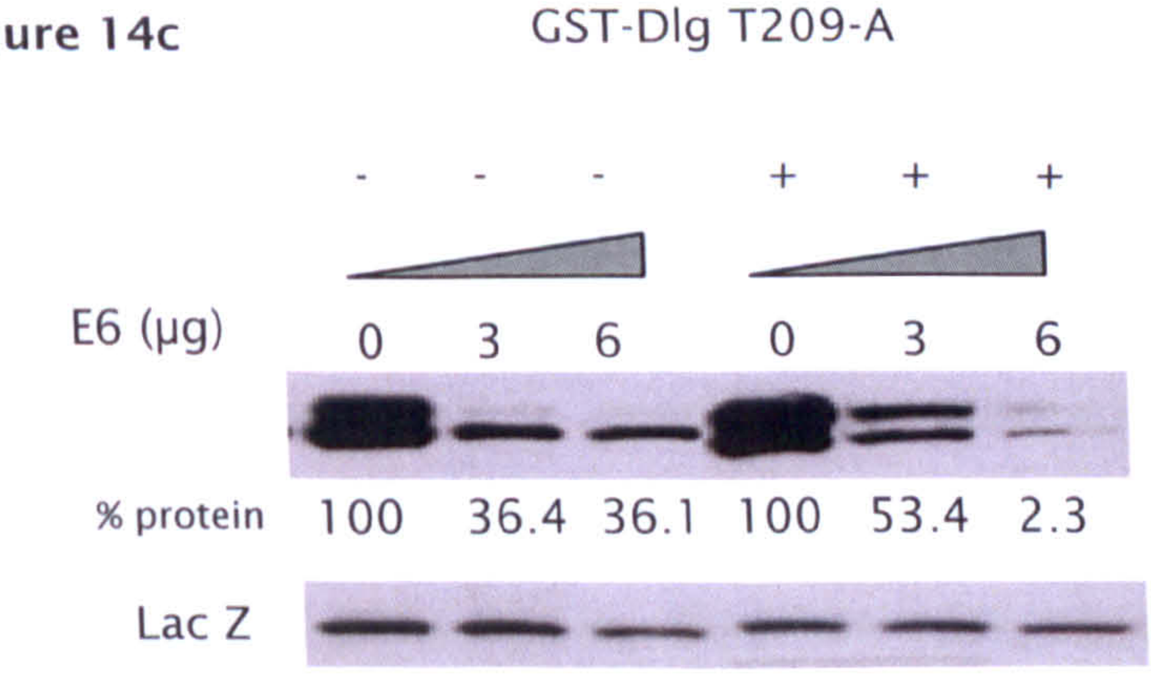


Figure 14: Identification of residues on hDlg conferring enhanced susceptibility to E6 degradation.

HEK293 cells were transfected as before with a)GST tagged wild type dlg, and the b)S158A and c)S209A mutants together with increasing amounts of E6 expression plasmid. After 24hrs the cells were exposed to 0.3M sorbitol as indicated and the proteins were extracted and analysed by western blotting with anti-GST antibody. The percentage of hDlg was quantified using densitometry. LacZ is included as a transfection control.

contrast, mutant S158, shown in figure 14b, which is still susceptible to E6 induced degradation, is however, unaffected by the addition of sorbitol. These results demonstrate that phosphorylation on residue S158 of Dlg is responsible for increasing Dlg susceptibility to E6 induced degradation under conditions of osmotic shock.

Part 2: The Phosphorylation of Dlg during the cell cycle

Cell cycle regulation of hDlg localisation and phosphorylation

Many kinases have been implicated in the regulation of the cell cycle, including the cdks, the polo-like kinases (PLKs) and the mitogen-activated protein kinases (MAPKs) to name a few. In many cases they function regulating the activity of their various substrates. Previous studies have also shown that Dlg can act negatively to suppress G0/G1 to S phase progression (Ishidate et al., 2000, Matsumine et al 1996), and that it is hyperphosphorylated during the M phase of the cell cycle (Gaudet et al., 2000). In addition, there are several reports detailing different patterns of expression of Dlg, including the presence of cytoplasmic, cytoskeletal-bound and nuclear forms of the protein, some of which are indeed regulated by different phosphorylation events. However no detailed studies have been performed to attempt to link these apparently diverse aspects of Dlg localisation and function with the cell cycle. Therefore we performed a series of studies to investigate the pattern of Dlg expression during the course of the cell cycle. To do this we first analysed the pattern of Dlg expression in asynchronously growing HaCaT cells. As can be seen from Figure 15a, there are diverse patterns of Dlg expression, with cytoplasmic, diffuse and membrane bound forms, as well as structures such as the midbody and the mitotic spindle clearly visible (see insets), suggesting potentially different patterns of hDlg expression, depending upon the phase of the cell cycle.

The specificity of the assay was verified using HaCaT cells stably expressing a shRNA against Dlg (Courtesy Massimi P.). As can be seen from Figure 15b these cells are devoid of detectable Dlg protein and the anti-Dlg antibody used shows no signal in these cells, confirming that the different patterns of expression observed in Figure 15a are indeed due to specific changes in the pattern of Dlg expression. To investigate this further we then proceeded to analyse the pattern of Dlg expression in HaCaT cells that had been synchronised following an Aphidicolin block. The aphidicolin block synchronises cells at G1/S and was used for cell synchronisation as opposed to the traditional use of the double thymidine block, as the double thymidine block does not work in cells demonstrating an intact p53 response, such as HaCaT cells. The cells were then harvested at specific time points and the patterns of Dlg expression assessed by western blotting (Figure 15c). The stage of the cells in the cell cycle was confirmed by performing a FACS analysis (Figure 16). As can be seen, at 5 hours (S phase) and 8 hours (M phase), slower migrating forms of Dlg were observed (Figure 15c). The lower panel showing a phosphatase assay verifies that these slower migrating forms are indeed phosphorylated forms of hDlg. Cyclin B is used as a control to verify the synchronisation, as can be seen by its accumulation in the stages leading to the M phase and its decline thereafter. However it must be noted that residual amounts of the protein are observed in the G1 fraction, suggesting that the synchronisation is not 100% efficient. To observe changes in patterns of localisation, cells were released and fixed at different times in a similar manner as above, but were then analysed for the pattern of Dlg expression by immunofluorescence. The results obtained are shown in Figure 17. In the G1 population Dlg is highly membrane-bound. However it progressively becomes less junctional and much more cytoplasmic as the cells progress through the cell cycle with a detectable amount of nuclear staining becoming evident as the cells become more rounded as they approach M phase. The protein shows a startling relocation to the mitotic spindle at mitosis and it finally accumulates dramatically at the midbody

Figure 15a

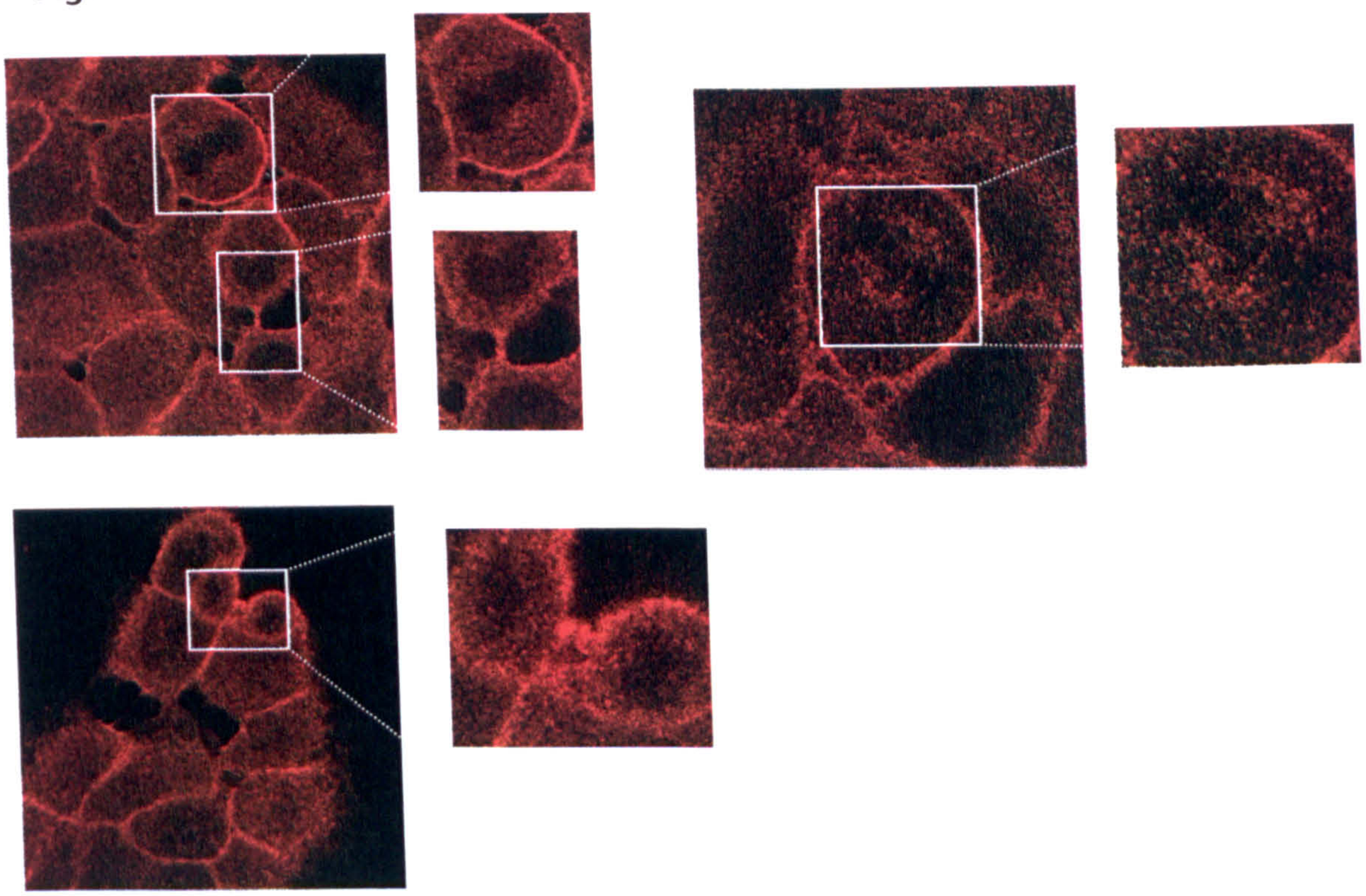


Figure 15b

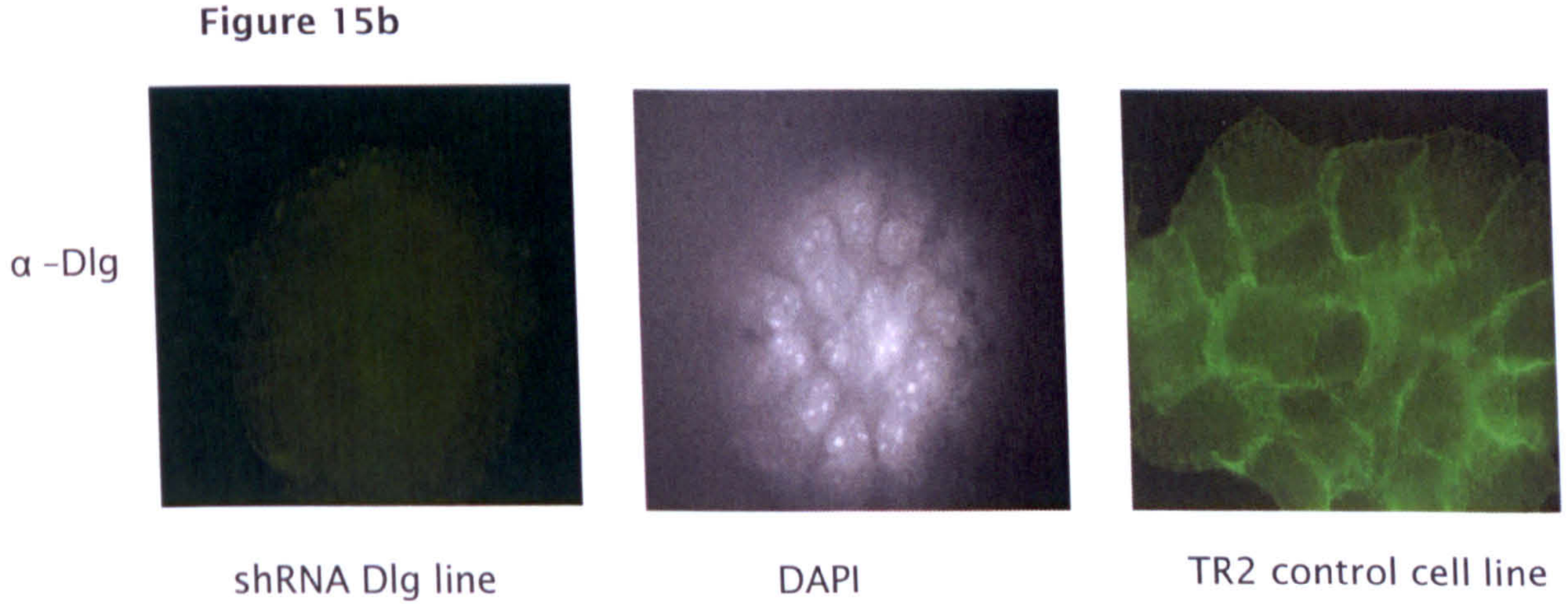


Figure 15 : Different patterns of Dlg expression in asynchronously growing cells. Panel a) Immunofluorescence analysis of Dlg expression was performed on HaCaT cells using the anti-Dlg monoclonal antibody 2D11. Various localisations of the protein can be seen in the two examples, including at sites of cell contact, diffuse cytoplasmic, nuclear excluded and also to the mitotic spindle at mitosis and to the midbody at cytokinesis (insets show the midbody and spindle). Panel b) The specificity of the staining patterns seen in (a) with the anti-Dlg antibody was verified by immunofluorescence analysis of Dlg expression in a HaCaT cell line stably transfected with an shRNA against Dlg (left hand panel). Also shown is the DAPI staining (middle panel) for the shDlg cells to show the nuclei and for comparison Dlg expression in a control cell line (right hand panel) stably transfected with empty vector.

Figure 15c

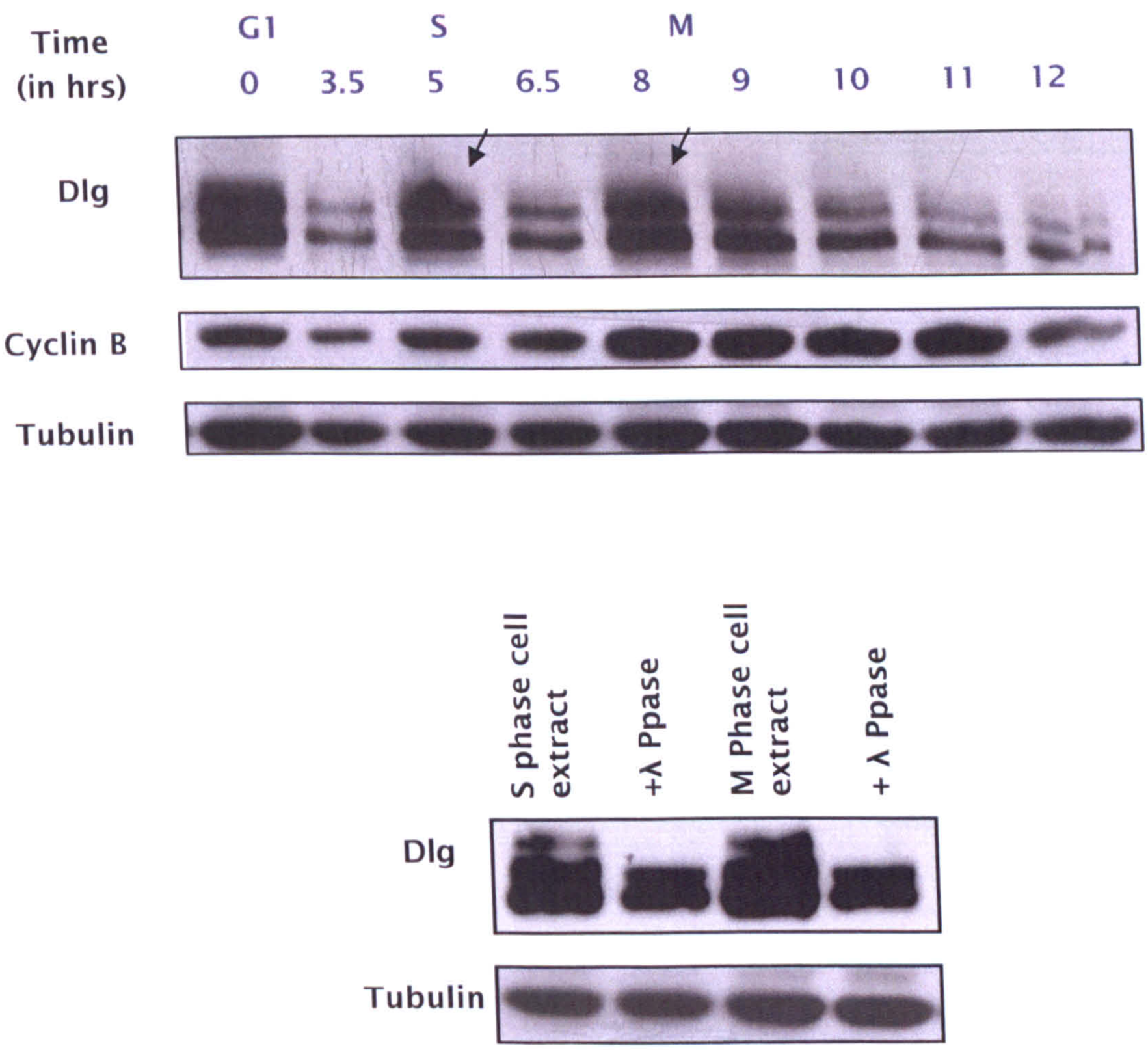


Figure 15 (contd) Changes in the pattern of Dlg expression during the cell cycle

c) Western blot depicting the regulation of Dlg through the cell cycle after an aphidicolin release. Note the decrease in protein levels in the time points preceding and succeeding the S and the M phases. An increase in the higher migrating forms of the protein is observed at 5 and 8 hours.

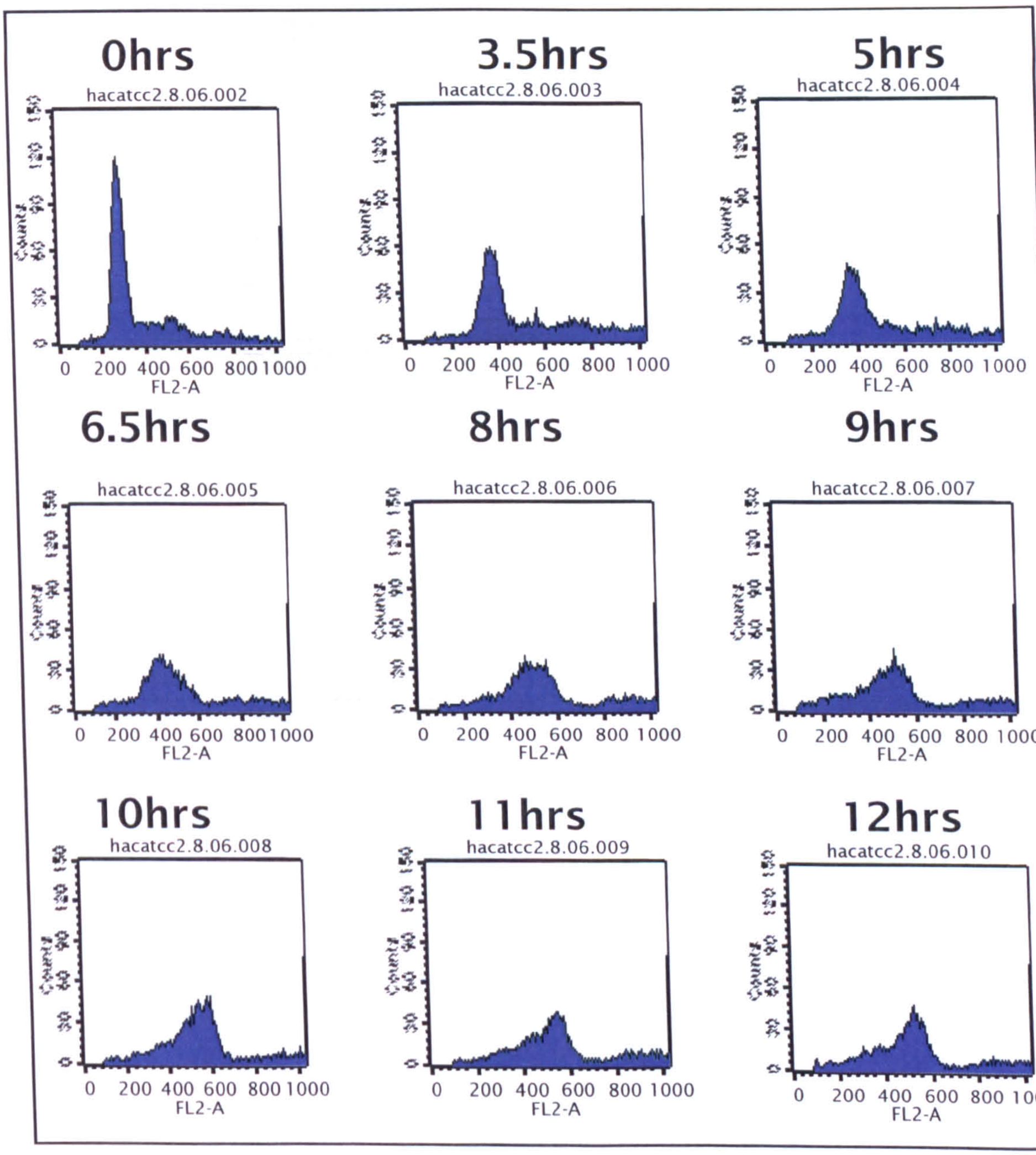


Figure 16 : FACS analysis showing cell cycle progression in HaCaTs following the aphidicolin block and release.

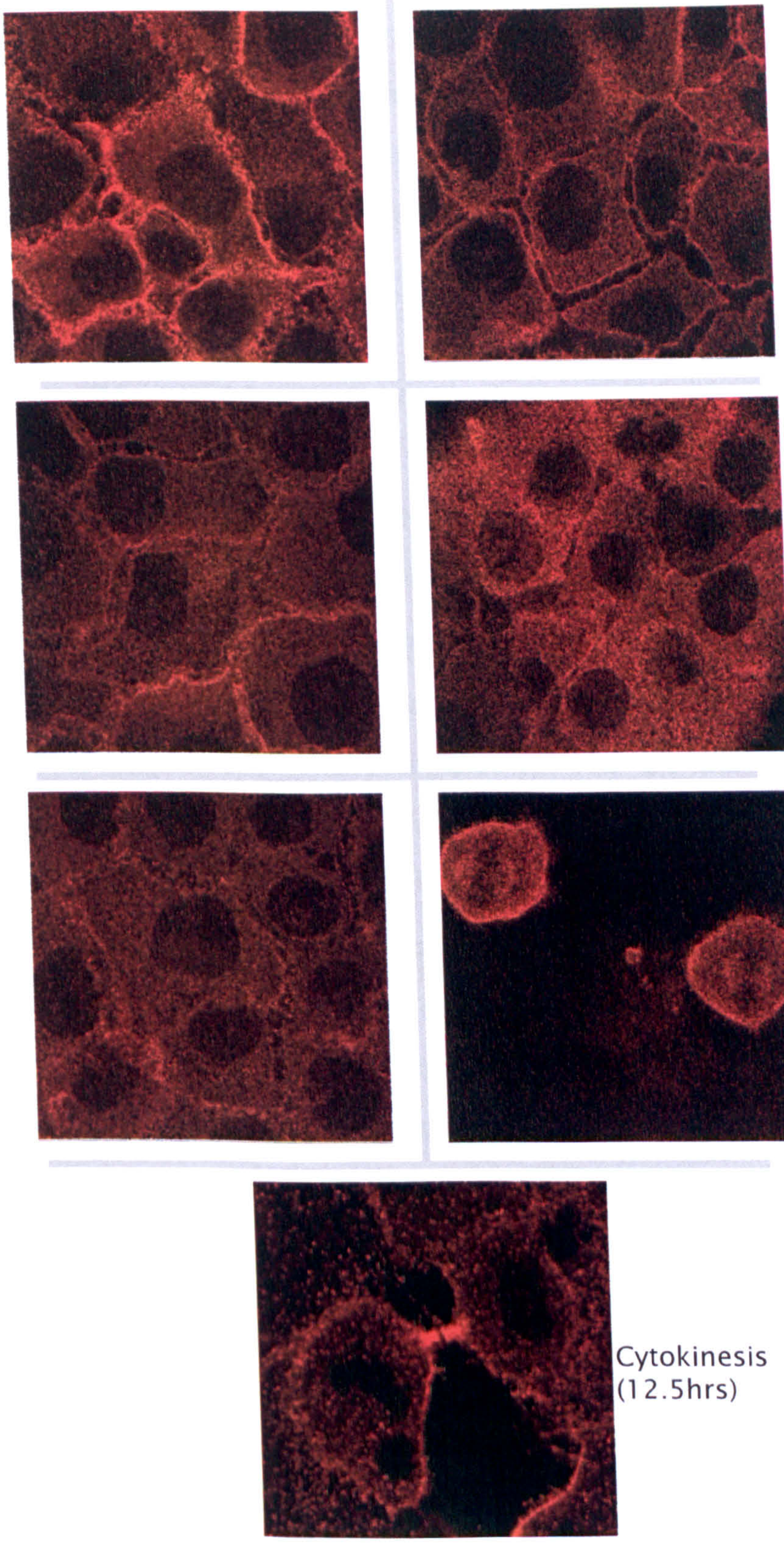


Figure 17 : Immunofluorescence analysis of Dlg expression during different phases of the cell cycle following arrest with aphidicolin (G1, 0 hrs) and subsequent release through to cytokinesis (12.5hrs).

during cytokinesis, in agreement with previous studies (Massimi et al., 2003). Taken together, these data suggest that Dlg is relocalised to different cellular compartments during different phases of the cell cycle.

hDlg is phosphorylated by cdk1 and cdk2 on serine 158 and serine 442

The only reported kinase potentially involved in the M phase phosphorylation of Dlg is the PDZ Binding Kinase (PBK) (Gaudet et al., 2000). Since we found that Dlg exhibits different patterns of expression both with respect to migration and localisation, during all of the cell cycle, we hypothesised that other kinases may have roles to play in this. Two candidates for this are the cyclin dependent kinases 1 and 2 (cdk1 & cdk2). In order to investigate whether Dlg could be a substrate for these kinases, we first performed in vitro phosphorylation assays with purified enzymes and a purified GST-Dlg fusion protein. The GST-Dlg fusion protein was washed in kinase buffer twice and then incubated with purified cdk1 or cdk2 along with radiolabelled ATP at 30°C for 20 minutes. The proteins were then washed extensively, resolved using SDS-PAGE and subjected to autoradiography. As Figure 18 shows, Dlg is phosphorylated in vitro by both cdk1 and cdk2, although it is generally a better substrate for cdk2 than for cdk1.

The next aim was to map the region(s) and possibly the residue(s) which is/are phosphorylated by the two kinases. To do this, we used constructs coding for various deletion mutants of GST-tagged Dlg were analysed (Gardiol et al., 2002). The mutants used separately spanned the entire sequence of the protein: the N terminus, the 3 PDZ domains and finally the C terminal region including the SH3 and GUK domains, as shown in Figure 19a. The in vitro kinase assays were carried out as described above, with purified cdk1 and cdk2 (Figures 19b and 19c respectively).

As can be seen, the results map the major sites of phosphorylation to residues within the N terminus and the 3PDZ region of Dlg. No detectable, or at best marginal, phosphorylation of the C terminal fragment was obtained with cdk1 and cdk2 respectively.

The Dlg amino acid sequence was then scanned for probable CDK consensus sites (S/T-P-X-R/K, where X = any amino acid) using the scansite software (http://scansite.mit.edu/), and two perfect consensus sites were found containing serine residues at 158, which lies in the N terminus, and at 442, which lies between PDZ domains 2 and 3. On analysing the human, mouse, rat, *Drosophila* and *Caenorhabditis elegans* Dlg sequences (Figure 20), it was found that these serines and the corresponding consensus sites are perfectly conserved among the mammals and vertebrates, but intriguingly they were not conserved in *Drosophila* and *C. elegans*. This suggests that these potential phosphorylation sites have roles in Dlg function in more developed species, but that different pathways regulate Dlg in lower organisms.

To verify that these two potential sites are indeed phosphorylated by cdk1 and cdk2, we mutated serines 158 and 442 to alanines both separately and together, to block phosphorylation on these residues. The mutants expressed as GST fusion proteins were then subjected to in vitro phosphorylation assays with purified cdk1 and 2. The results obtained are shown in Figures 21a and 21b. As can be seen, when either residue alone is mutated Dlg was still phosphorylated by both kinases with almost wild type efficiency. Only when both sites are mutated is phosphorylation by cdk1 and 2 abolished. This suggests that both serines are equally recognised by the CDKs as being phospho-acceptor sites. From figure 19b and 19c, it is observed that both kinases seem to phosphorylate the GST-N-terminal fragment of Dlg more efficiently than the full-length protein, suggesting that serine 158 may be a better

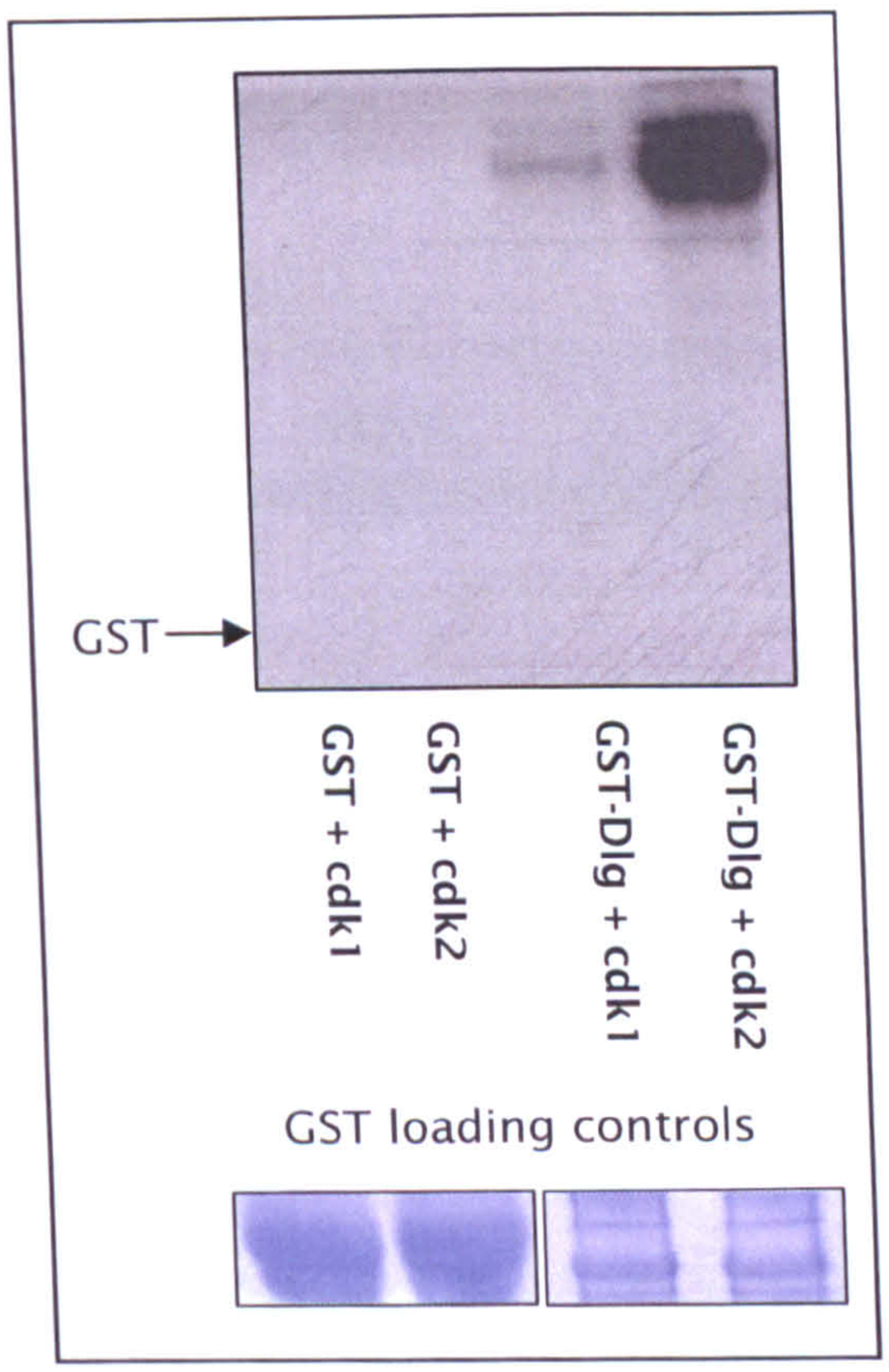


Figure 18 : Dlg is phosphorylated by cdk1 and cdk2 in vitro.

GST-Dlg was incubated with purified cdk1 and cdk2 plus radio-labelled ATP for 20min, and phosphorylation of Dlg ascertained by SDS-PAGE and autoradiography (upper panel). The lower panel shows the Coomassie stained gel showing the control GST and the GST-Dlg fusion protein.

Figure 19a

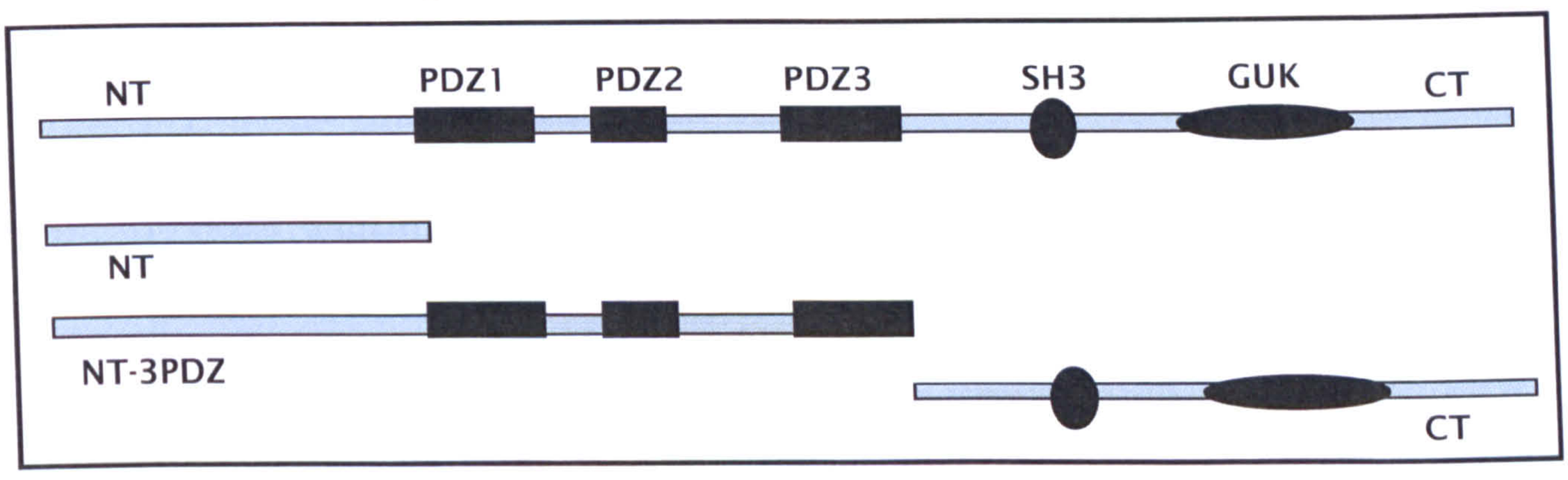


Figure 19b

Figure 19c

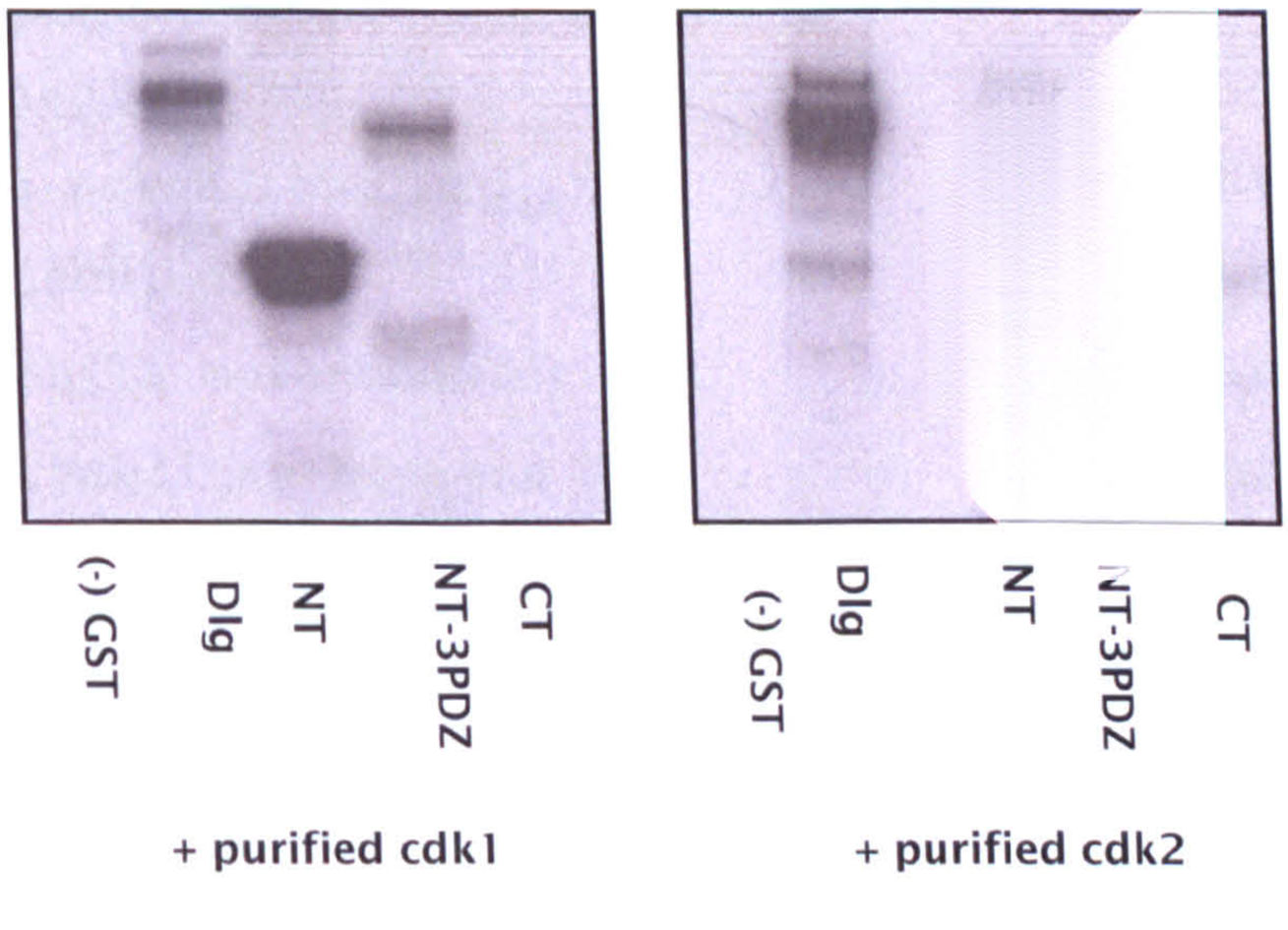


Figure 19 Identification of regions of Dlg that are phosphorylated by cdk1 and cdk2

Panel a) shows a schematic of the different expression constructs of Dlg used in the analysis. The purified fusion proteins were then incubated with purified cdk1 (panel b) and cdk2 (panel c) together with radiolabelled ATP. Phosphorylated proteins were analysed by SDS PAGE and autoradiography.

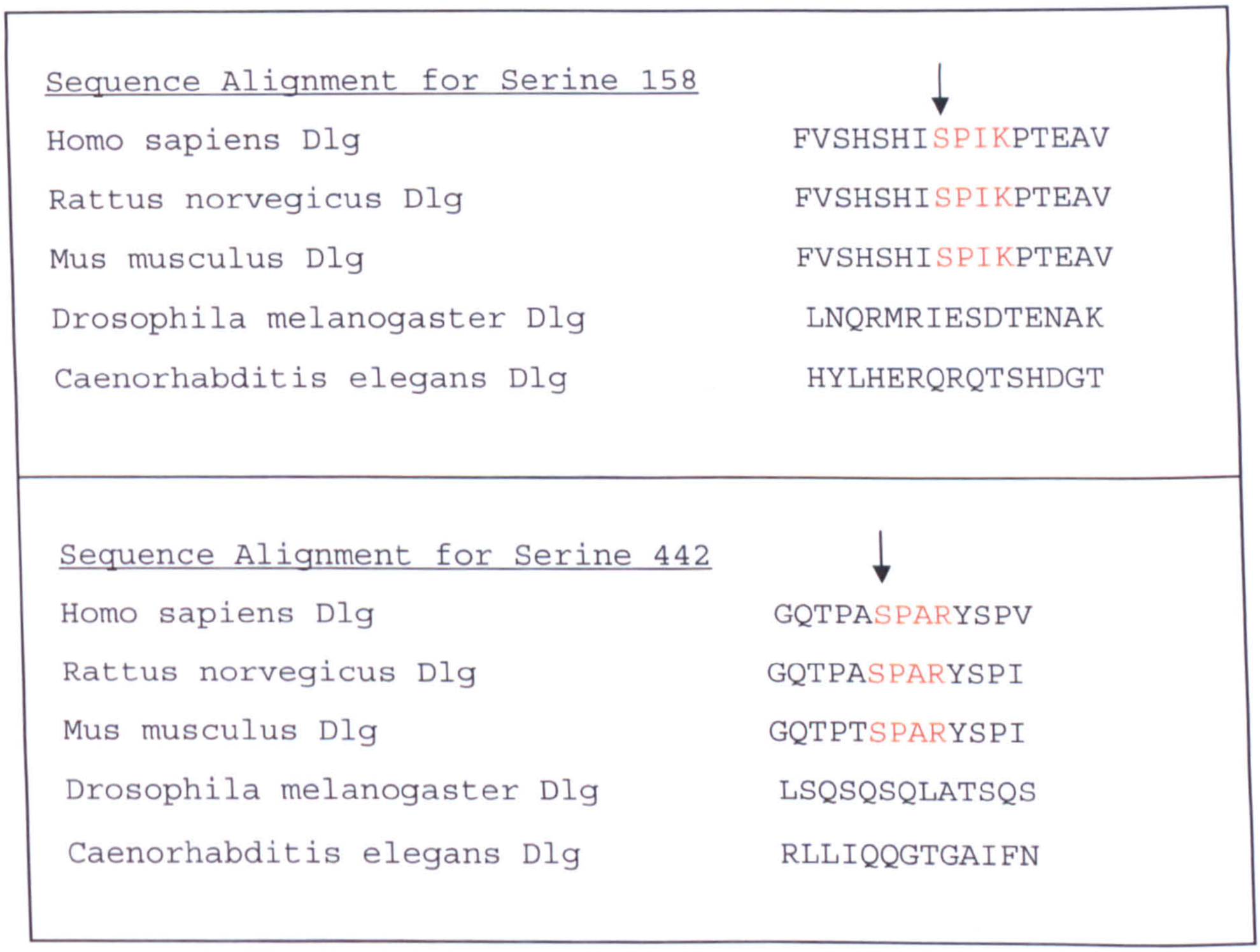
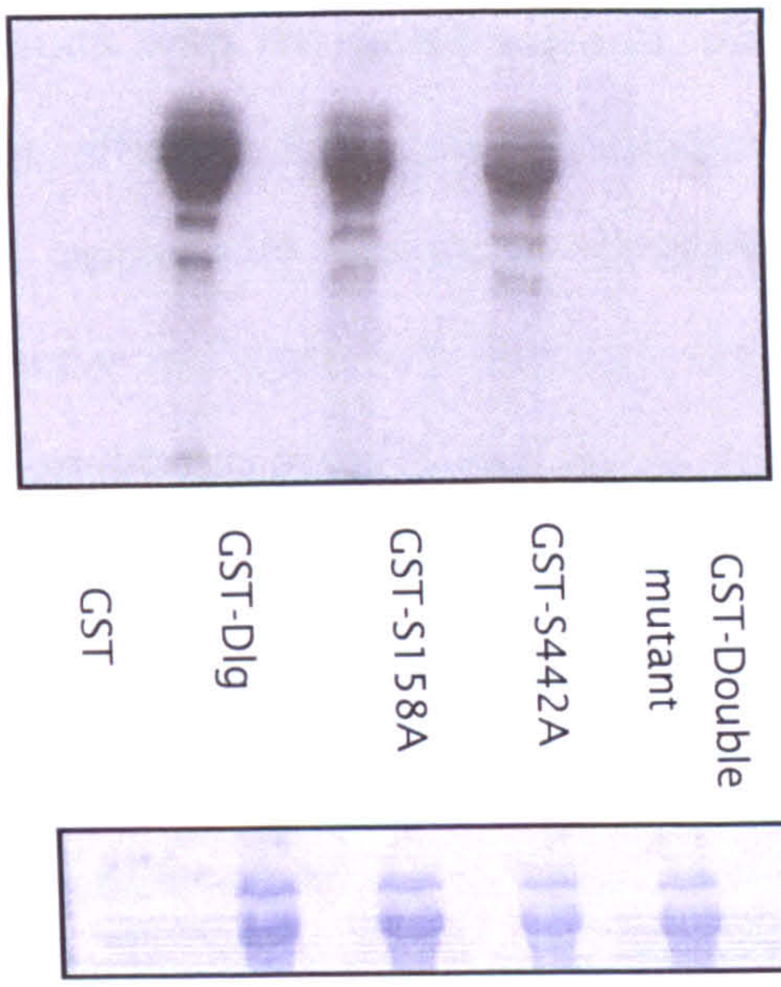


Figure 20 Comparison and sequence alignment of the regions of Dlg containing the consensus cdk phosphorylation sites in humans, mice, rats, *Drosophila* and *C.elegans*. The sequences were obtained from the ExPASy proteomics server (http://www.expasy.org/).

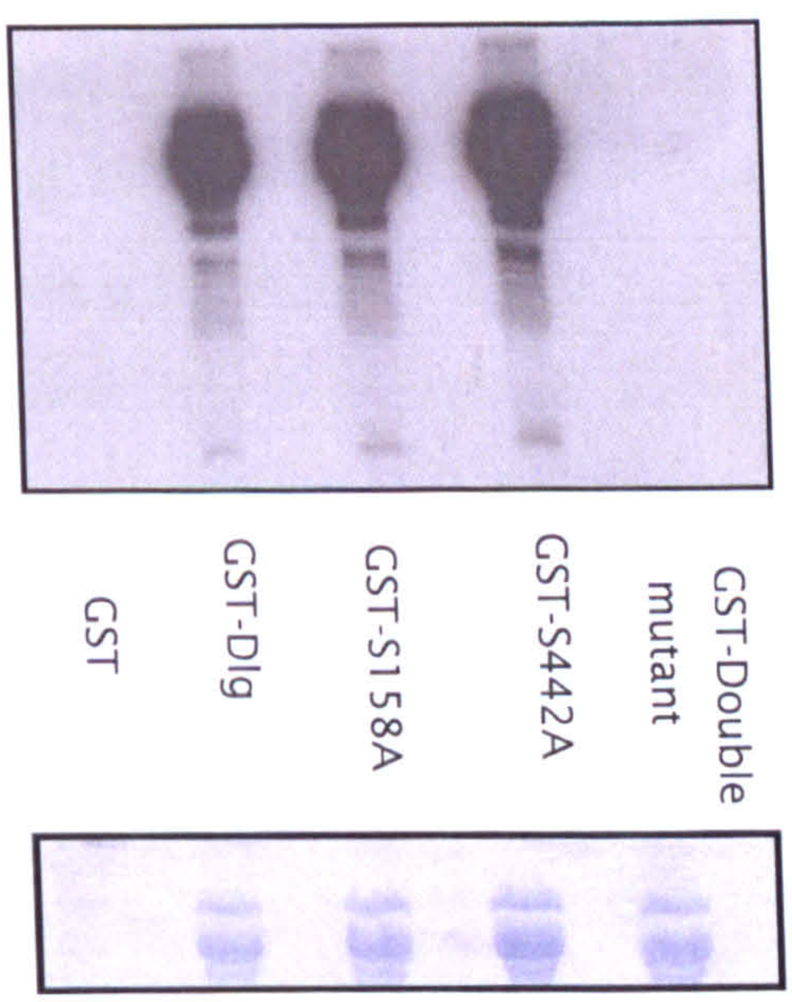
Figure 21a



+ purified cdk1

Coomassie Staining of Gels

Figure 21b



+ purified cdk2

Coomassie Staining of Gels

Figure 21 : Dlg mutated at serines 158 and 442 cannot be phosphorylated by the CDKs.
GST-tagged Ser158A, Ser442A and the double mutant in which both serines are mutated to alanine were subjected to in vitro kinase assays with cdk1(panel a) and cdk2 (panel b) respectively, and analysed by SDS PAGE and autoradiography (upper panels). The lower panels show the Coomassie stained gels and confirm equal amounts of protein loading. Phosphorylation by both kinases is lost in the mutant lacking both serines.

substrate. But the results with the point mutants clearly show that both sites are equally important for efficient CDK phosphorylation of the full length protein, suggesting that the apparently enhanced phosphorylation of the N-terminal fragment that we observe in Figure 19b and 19c, might be due to a change in the structural conformation of the protein due to its truncation, thereby making the phospho-acceptor site more available to the kinase.

hDlg is found in CDK-containing complexes in vivo.

To investigate whether Dlg could complex with and be phosphorylated by CDKs in vivo, we first performed a series of co-immunoprecipitation experiments in HEK293 cells, immunoprecipitating endogenous cdk2 and then probing for endogenous Dlg. Figure 22a and 22b demonstrate that Dlg co-immunoprecipitates with cdk2 as well as the cdk1 cyclin subunit, cyclin B, although we were unable to detect an interaction by co-immunoprecipitation of Dlg and cdk1. This might be a reflection of the relative differences in the efficiency by which cdk1 and cdk2 can phosphorylate Dlg in vitro, with cdk2 phosphorylation of Dlg generally being much stronger. Therefore to ascertain whether the M phase phosphorylation of Dlg was in part mediated by cdk1, a different approach was taken. The cells were treated for 18 hours with the microtubule-depolymerising agent Nocodazole, to block cells in mitosis, which was confirmed by FACS analysis (Figure 23). The cells were then treated with the cdk-specific inhibitor Roscovitine for three hours and Dlg expression was analysed by western blotting. Roscovitine is an inhibitor that is active both against cdk1 as well as cdk2, hence the Nocodazole block was performed to synchronise cells in M phase and to ensure that the only active CDK that roscovitine will inhibit in those cells is cdk1. The results obtained are shown in Figure 23. As can be seen, a number of slower migrating forms of Dlg are present in Nocodazole-arrested M phase cells, indicating that multiple phosphorylation events take place on Dlg in the presence of Nocodazole. Intriguingly, the slowest migrating form disappears following treatment

with Roscovitine, demonstrating that at least one of the M phase phosphorylation events on Dlg is mediated by cdk1. For comparison, asynchronously growing cells were likewise treated with Roscovitine and the effects on Dlg expression analysed by western blotting. As can be seen from Figure 23 there is also a significant decrease in the intensity of the slower migrating phosphorylated form of Dlg (Massimi et al., 2006), further supporting the notion that Dlg is a substrate for CDKs in vivo.

Phosphorylation on serines 158 and 442 enhances nuclear expression of hDlg

To investigate the role of the CDK phosphorylation events on Dlg function we first analysed the effects of mutating these residues upon the pattern of Dlg expression. To do this a series of HA-tagged Dlg mutants were generated with the serines mutated to Alanine (A), to block phosphorylation or Aspartic Acid (D) to mimic phosphorylation - Ser-158A and Ser-158D, Ser-442A and Ser-442D, Ser-158Ser-442AA and Ser-158Ser-442DD. In order to verify that the different Dlg mutants were expressed, 293 cells were transfected with the different expression plasmids and after 24hrs protein levels were ascertained by western blot analysis. The results obtained are shown in Figure 24. Interestingly, the wild type Dlg migrates as two distinct forms on the gel, the slower of which is more heavily phosphorylated (Caruana and Bernstein 2001; Massimi et al., 2006), whilst the double A mutant co-migrates with the faster migrating band, and the double D mutant co-migrates with the slower migrating phosphorylated form of Dlg. In addition, the single point mutations at S158 have essentially a similar phenotype, demonstrating that the presence or absence of a single acidic residue at this position has a profound effect on the overall conformational structure of Dlg, and that a single acidic residue at 158 is sufficient to account for the slower migrating form of Dlg.

Figure 22a

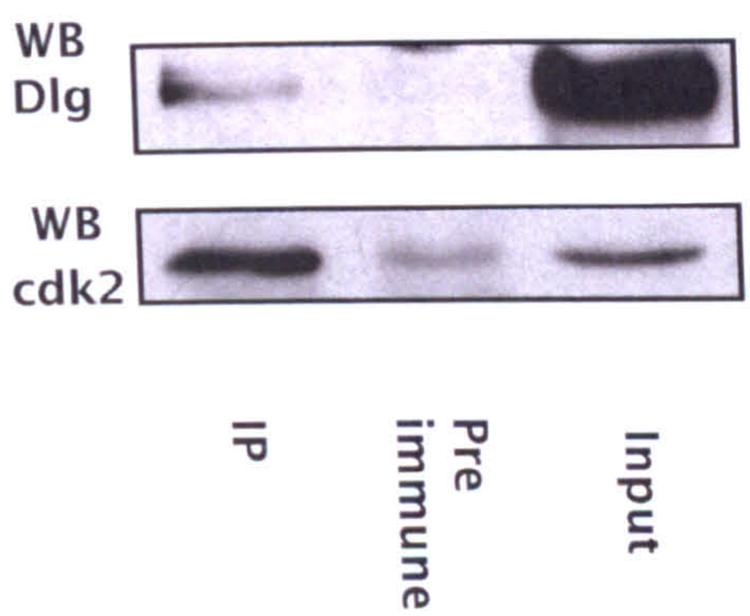


Figure 22b

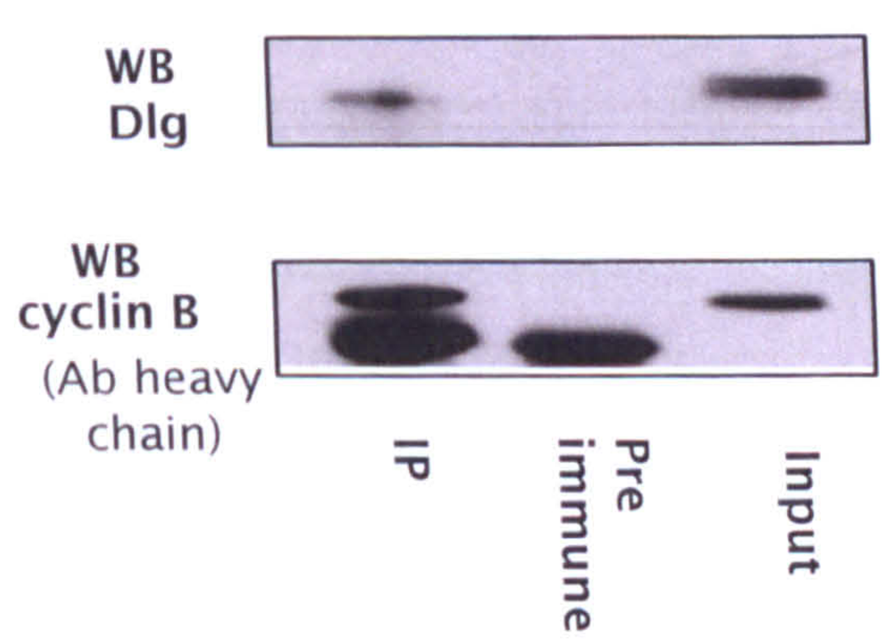
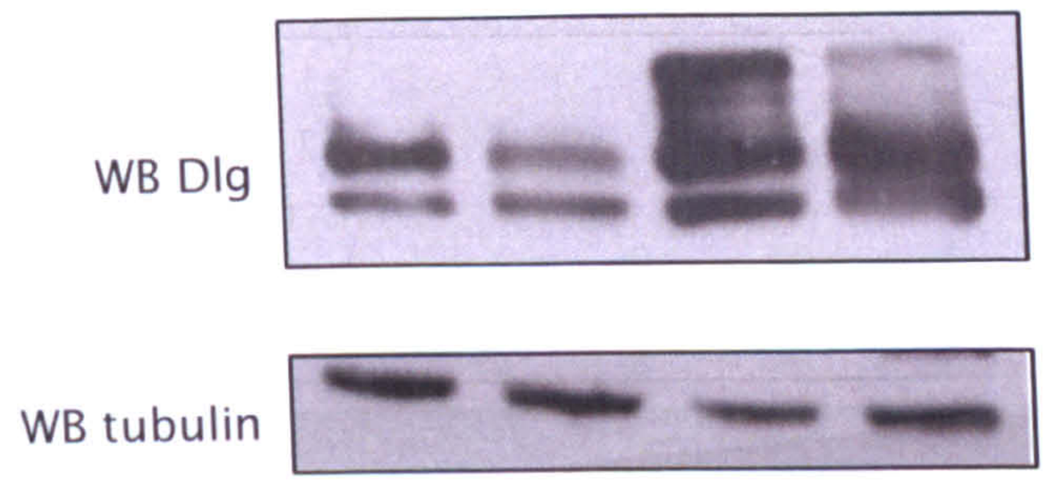


Figure 22 : Dlg associates in vivo with cdk1 and cdk2.

Asynchronously growing 293 cells were extracted with E1a buffer and extracts immunoprecipitated with anti-cdk2 (a), anti-cyclin B (b) antibodies (IP) or pre-immune antibody as indicated. Co-precipitated Dlg was detected by western blot analysis with anti-Dlg monoclonal antibody (upper panels) and immunoprecipitated cdk2 and cyclin B detected also be western blotting (lower panels). Twenty percent of each cell extract was used as input control.

Figure 23



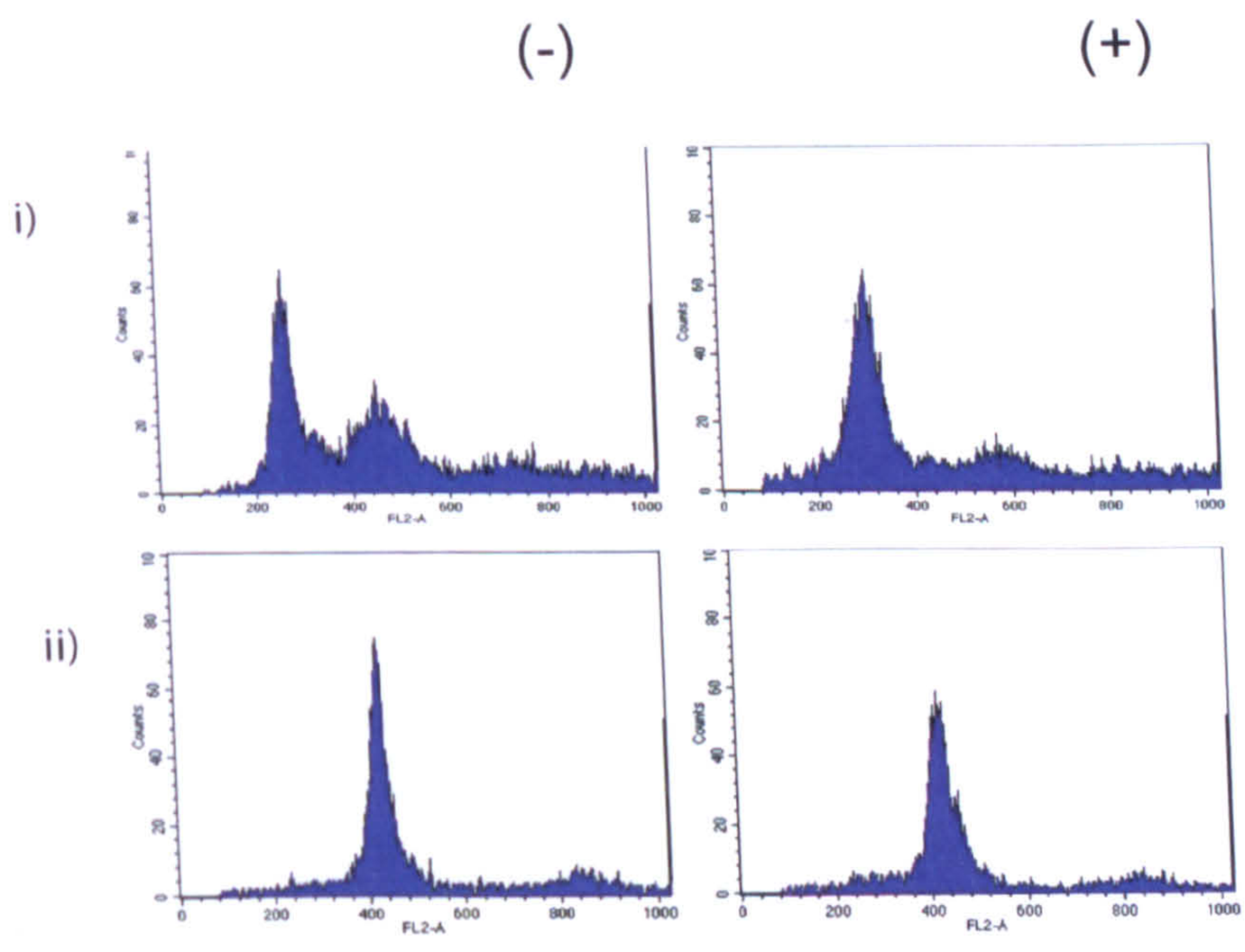


Figure 23 : Dlg associates in vivo with cdk1 and cdk2

HaCaT cells were cultured for 18hrs with (+) or without (-) nocodoazole for 18hrs and were thereafter treated with 50μM Roscovitine (+ Rosco) for three hours to inhibit cdk1 activity. The pattern of Dlg expression was then analysed by western blotting with anti-Dlg monoclonal antibody. Note the decrease in the intensity of the slower migrating forms of Dlg in both the asynchronous and Nocodazole arrested cells following treatment with Roscovitine. FACS analysis showing i) asynchronous cells and ii) cells enriched in M phase, untreated (-) or treated (+) with roscovitine.

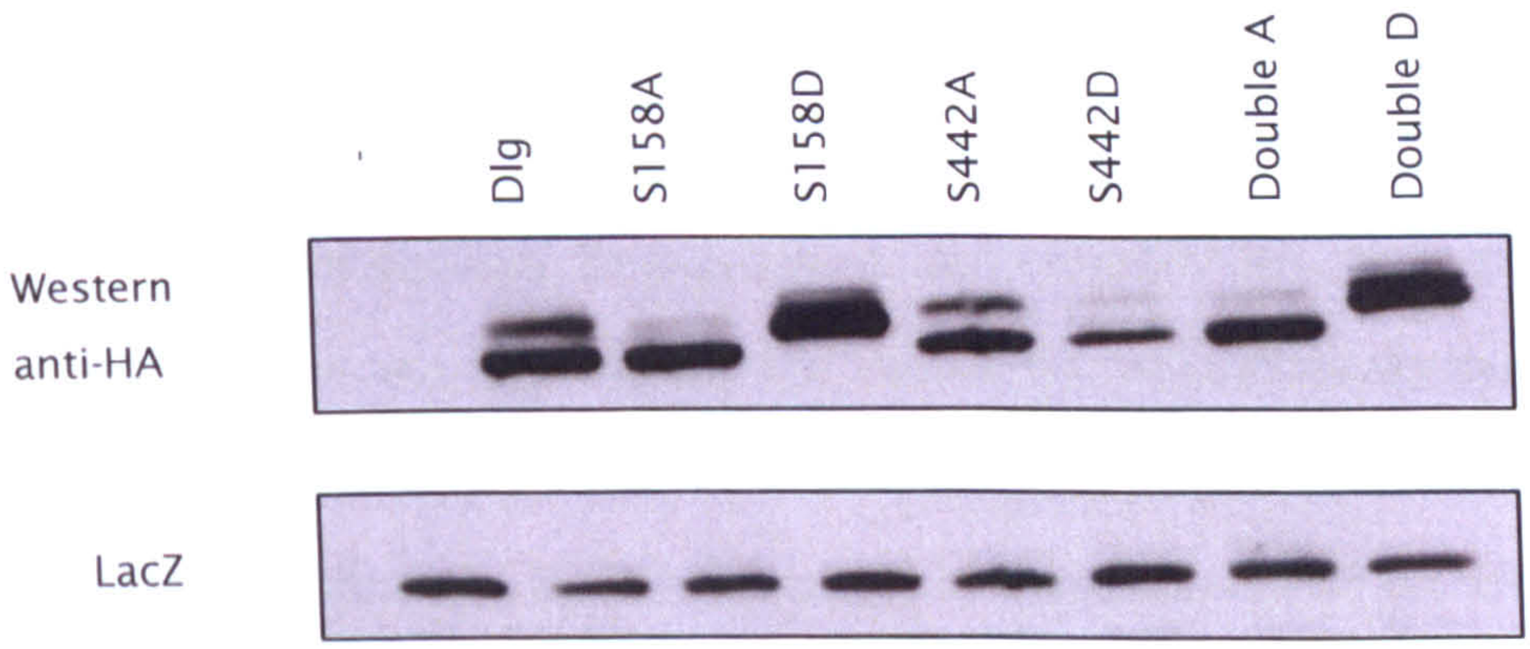


Figure 24 : The phospho-point mutants migrate differently from each other 293 cells were transfected with the different HA-tagged Dlg expression plasmids together with a beta-galactosidase expression plasmid and the pattern of Dlg expression analysed by western blotting with anti-HA monoclonal antibody (upper panel) and anti beta-galactosidase antibody (lower panel) as a marker for transfection efficiency.

We then proceeded to investigate the effects of the two double point mutations upon the pattern of Dlg expression in vivo. U2OS cells were transfected with the different expression plasmids and Dlg expression ascertained by immunofluorescence using anti-HA antibodies. The results obtained in Figure 25a show the predominant patterns of expression that are observed. Wild-type Dlg exhibits two distinct patterns of expression: a diffuse staining of the entire cell and a staining that excludes the nucleus. However, the point mutant Ser-158Ser-442AA shows a predominantly nuclear excluded pattern, while the Ser-158Ser-442DD shows a more diffuse pattern of staining. To more accurately quantify this, 300 cells were counted for each assay and the expression scored as diffuse or nuclear exclusion and the results are shown in the form of a graphical representation in Figure 25b, which further confirm the differential pattern of expression of the two mutant proteins.

To further demonstrate a direct link between cdk activity and the pattern of Dlg1 localisation, we analysed the pattern of Dlg1 expression in HaCaT cells in the presence and absence of the cdk inhibitor roscovitine. The results obtained are shown in Figure 25c where it can be seen that treatment with roscovitine induces an overall decrease in the intensity of Dlg1 staining and a more diffused cytoplasmic pattern of expression. However there is no apparent change in the ability of Dlg1 to localise to the midbody in cytokinesis, although we were unable to detect localisation to the mitotic spindle in the few dividing cells that could be detected. These results demonstrate that inhibition of cdk activity has a direct effect upon the pattern of Dlg1 expression.

hDlg protein stability and susceptibility to ubiquitination is in part determined by its phosphorylation on serine 158 and serine 442

The above results demonstrate that phosphorylation of Dlg on S158 and S442 can affect its cellular pattern of expression. We also noticed from Figure 24 subtle differences in the levels of expression of the different mutant forms of Dlg and, since phosphorylation of proteins has long been connected to their stability in the cell, we asked whether the phosphorylation events on these serines could affect Dlg protein stability. Cells were transfected with the different expression plasmids and after 24hrs further protein synthesis was blocked by treatment with cycloheximide. The levels of Dlg expression were then analysed over a period of 8hrs by western blotting and the results obtained are shown in Figure 26a. Strikingly, we found that the Dlg-AA mutant has a significantly reduced half-life in comparison with both wild type Dlg and the Dlg-DD mutant. The bands were also quantitated using Adobe Photoshop and the results are shown in the graph. This suggests that phosphorylation of the protein on these two residues increases its stability and affects the turnover of the protein. To further investigate this we also performed an in vivo ubiquitination assay. Cells were transfected with the HA-tagged Dlg expression plasmids together with a FLAG-tagged ubiquitin expression plasmid. As shown in Figure 26b, the inputs for all three proteins were approximately equal, however following immunoprecipitation of Dlg and western blot detection for ubiquitin we find that the wild type and the Dlg-AA mutant show a high degree of ubiquitination. In contrast, the Dlg-DD mutant has only a very low level of ubiquitination. These results suggest that the acidic status of the S158 and S442 residues can directly affect the levels to which Dlg is ubiquitinated which in turn affects the overall stability of the protein.

hDlg is phosphorylated in vivo in a cell cycle dependent manner on serine 158 and serine 442

The above data provides compelling evidence that Dlg can be a substrate for CDK phosphorylation on two different residues, and therefore suggests

Figure 25a

i) And ii) Dlg iii) Dlg-AA iv) Dlg-DD

Figure 25b

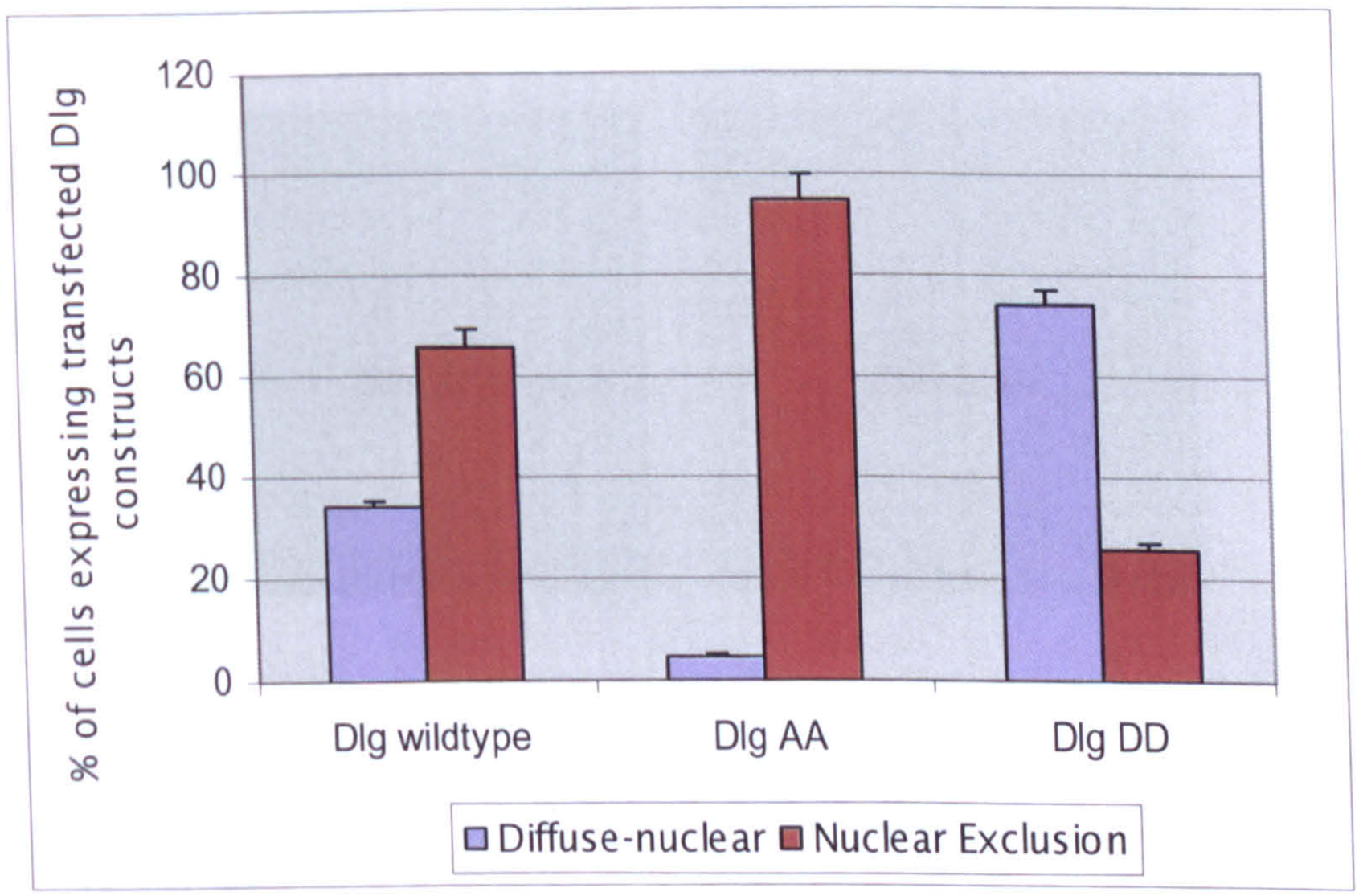


Figure 25 : The phospho-point mutants localise differently from each other

a) U2OS cells were transfected with i) and ii) Dlg wildtype, iii) Dlg-AA and iv) Dlg-DD mutants and the pattern of expression analysed by immunofluorescence using anti-HA antibodies. Note the two distinct patterns of expression observed with wild type Dlg compared with the predominant patterns seen with the two double mutants.
b) Quantisation of the percentage of cells showing the two predominant patterns of Dlg expression (diffuse/nuclear and nuclear exclusion) from a total of 300 cells transfected in each case.

Figure 25c

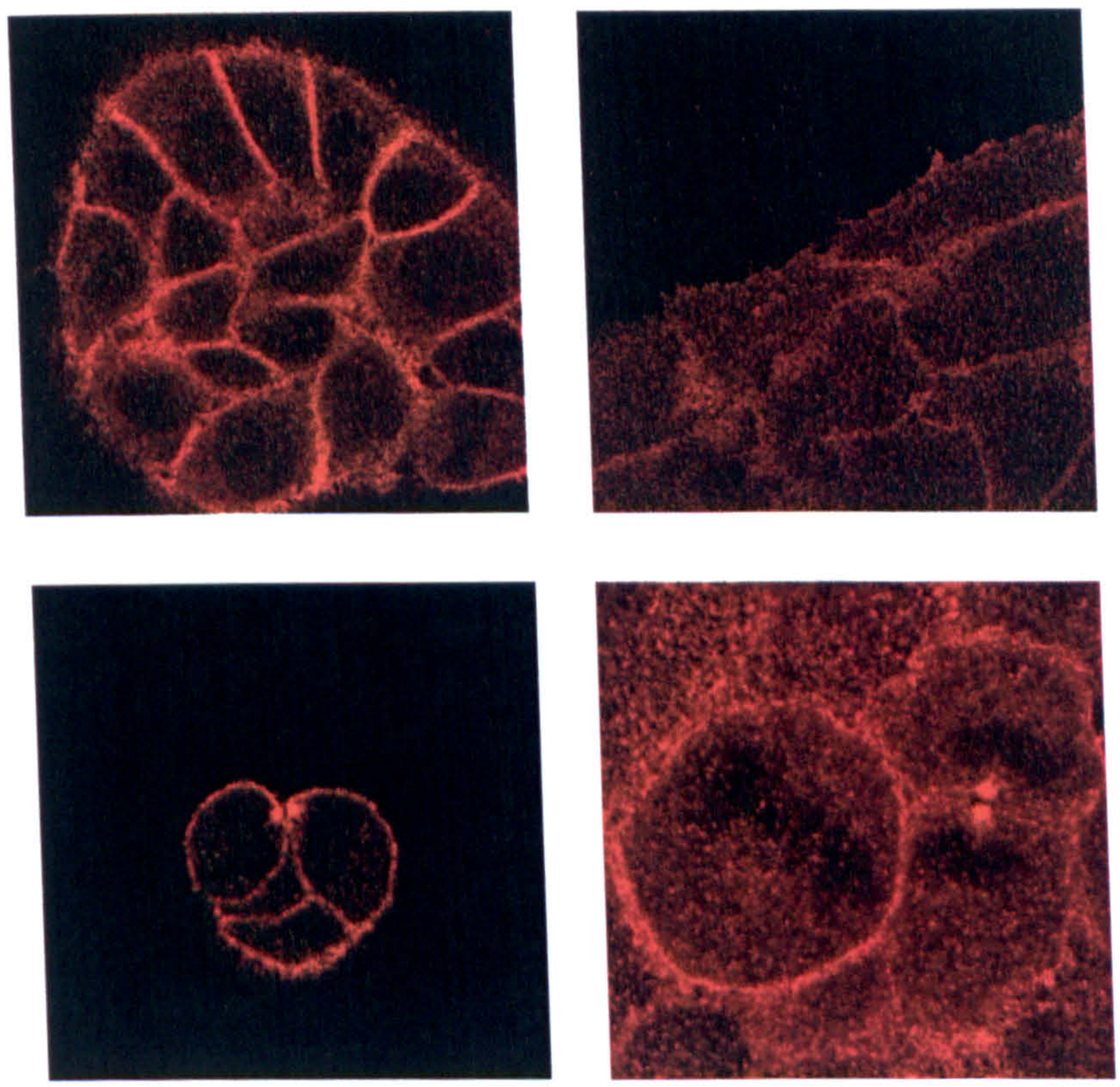
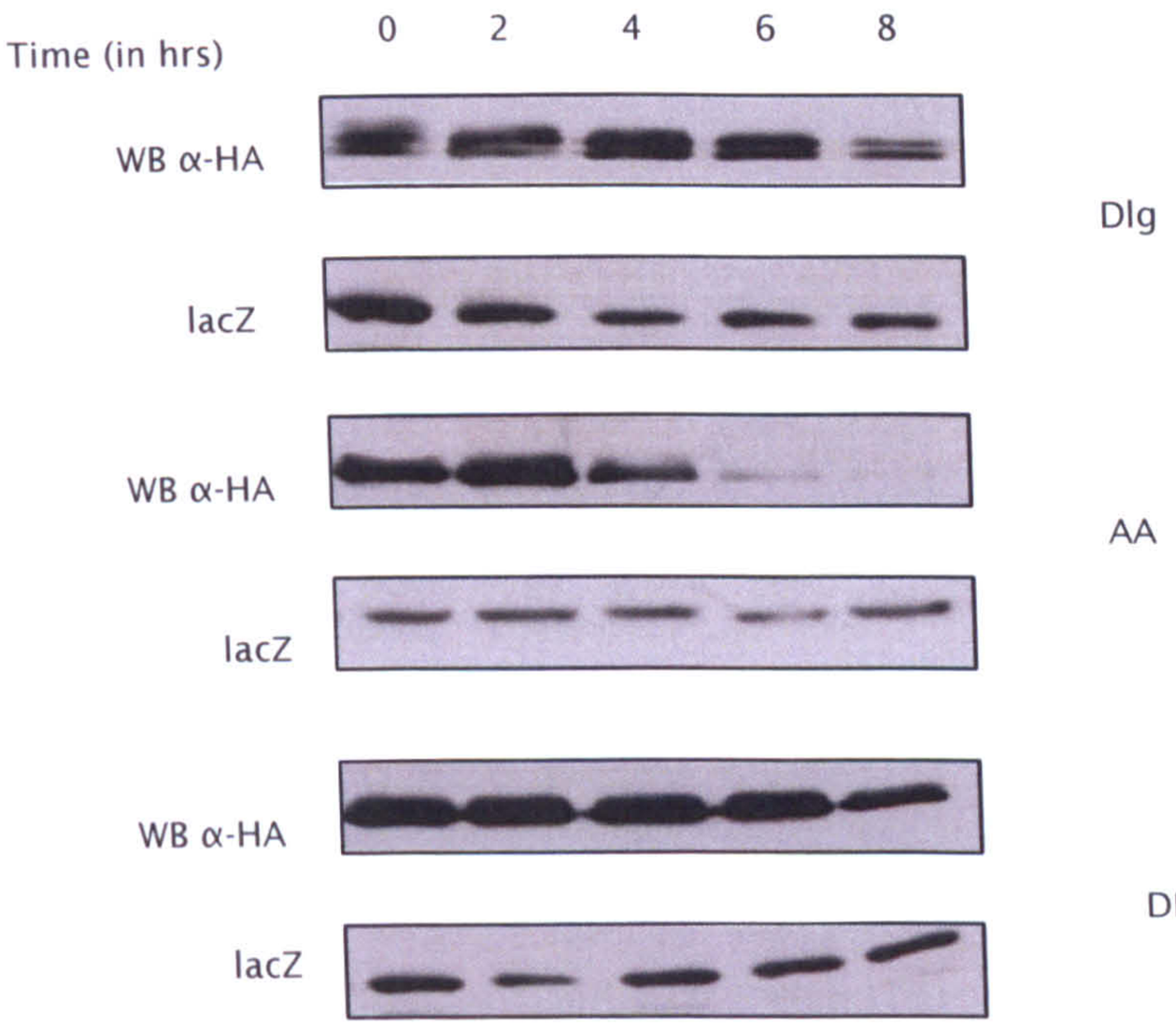


Figure 25

c) Immunofluorescence detection of endogenous Dlg1 in HaCaT cells in the absence and presence of roscovitine (50mM for 3 hours). Note the general decrease in the levels of Dlg1 and the more diffused cytoplasmic staining in the presence of the cdk inhibitor (upper panels), although there is no change in Dlg1 localisation to the midbody (lower panels).

Figure 26a



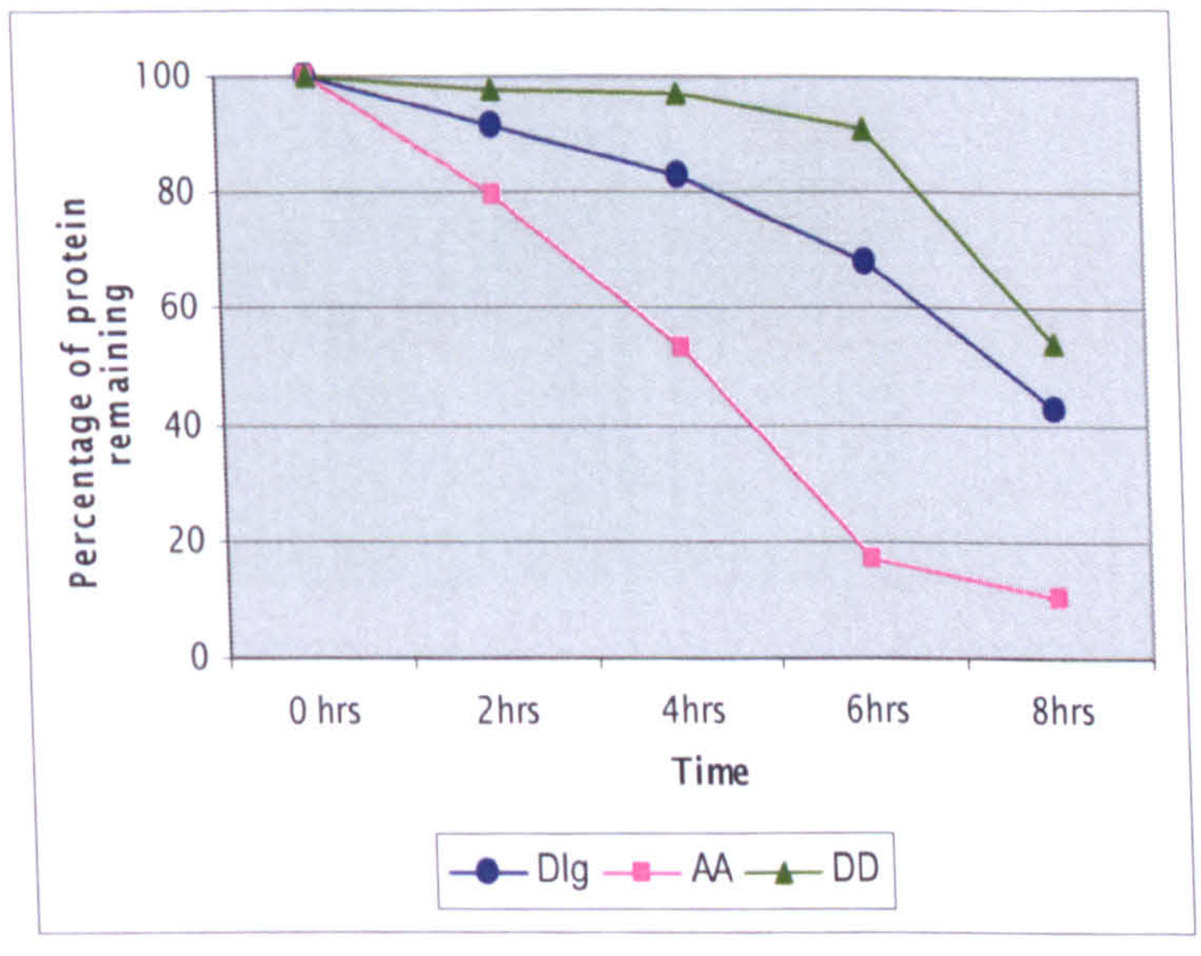
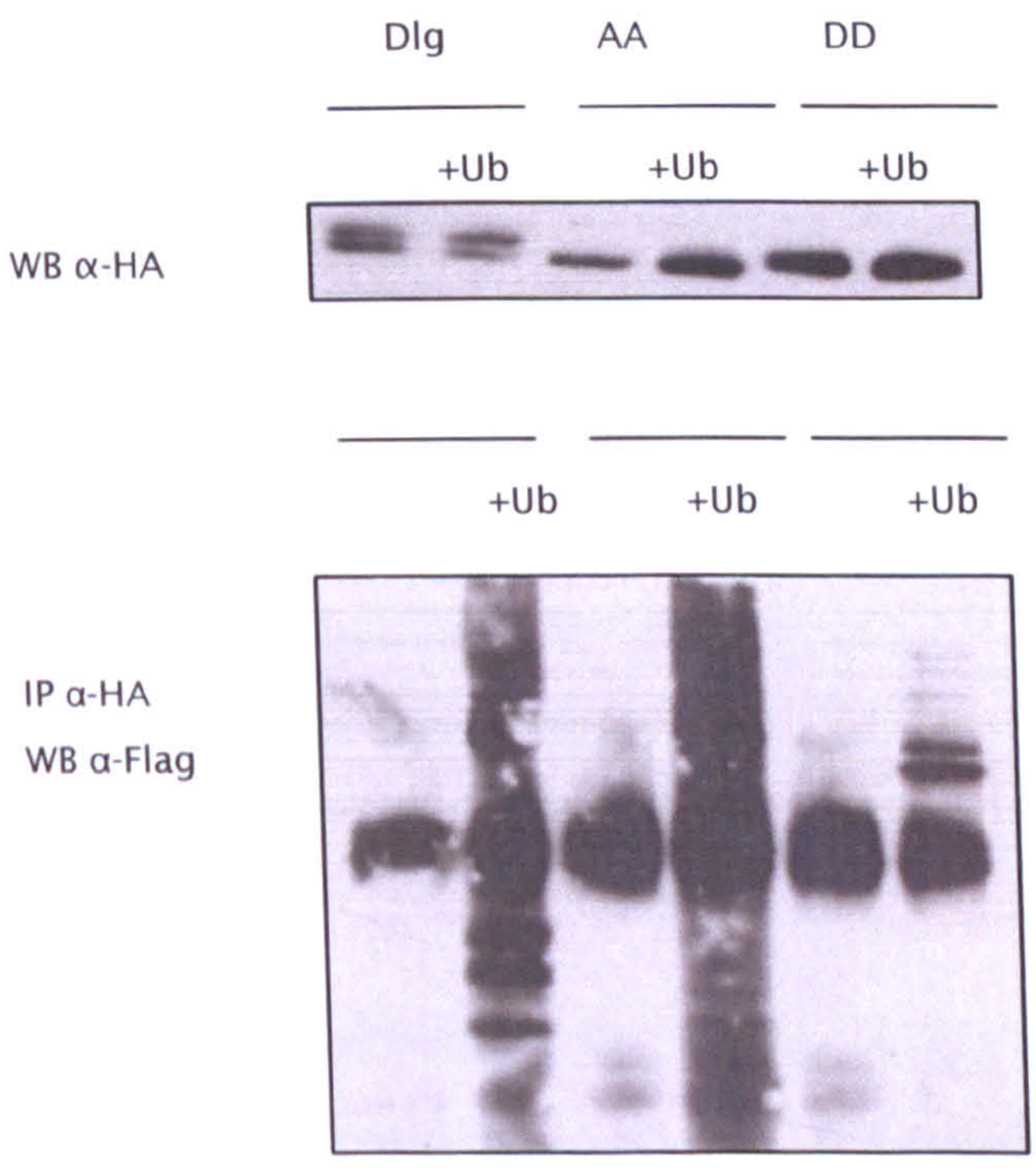


Figure 26 : The Dlg phospho-mimic mutant has a longer half life and is less susceptible to ubiquitination

a) 293 cells were transfected with the different HA-tagged Dlg expression plasmids together with a beta-galactosidase expression plasmid and after 24hrs the cells were treated with cycloheximide for the times indicated. Cells were then harvested and Dlg expression ascertained by western blot analysis with the anti-HA antibody (upper panels) and anti beta-galactosidase to control for transfection efficiency (lower panels). Densitometry was performed using Adobe Photoshop and the amount of protein at 0hrs was defined as 100%. The results obtained are shown in the graph.

Figure 26b



(continued) Figure 26 : The Dlg phospho-mimic mutant is less susceptible to ubiquitination

b) The different Dlg expression plasmids were transfected into 293 cells in the presence (+Ub) or absence of FLAG-tagged ubiquitin expression plasmid and the levels of Dlg expression first analysed by anti HA western blot. These extracts were then subjected to immunoprecipitation with anti-HA antibody and western blot detection with anti FLAG antibody to detect the presence of ubiquitin conjugates on Dlg. Note the high degree of ubiquitination with the wild type Dlg and the AA mutant, but very low levels of ubiquitination on the DD mutant despite readily detectable levels of Dlg expression in the input panel.

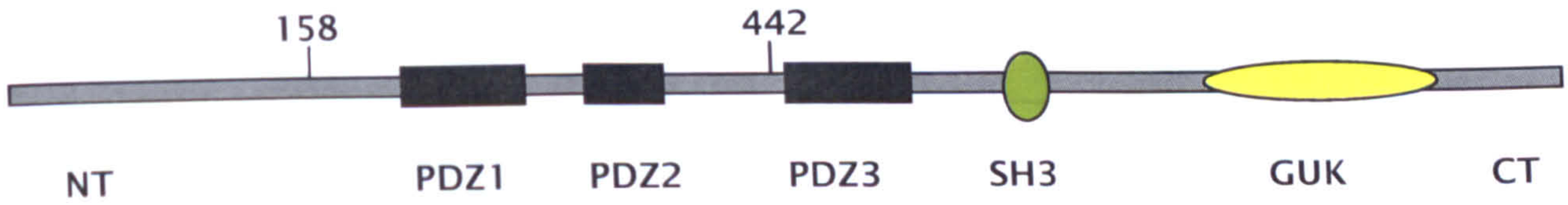
that phosphorylation on these sites will be cell cycle dependent. In order to formally verify this, we proceeded to generate rabbit polyclonal phospho-specific antibodies against phospho-S158 and phospho-S442. The peptide sequences shown in figure 27 were sent to Eurogentec Inc., for the production of the phospho-specific antibodies to serine 158 and 442. In order to confirm the specificity of these antibodies in western blotting, we first phosphorylated GST-Dlg fusion protein in vitro with purified cdk1 and cdk2 and cold ATP. The proteins were then subjected to gel electrophoresis and western blotting with the phospho-specific antibodies as well as with control anti-Dlg monoclonal antibody. As can be seen from Figure 28 the anti-Dlg monoclonal antibody recognises both the phosphorylated and non-phosphorylated forms of the protein with approximately equal affinity. In contrast, the phospho-specific antibodies to Serine 158 and Serine 442 each only recognise the cdk1 and cdk2 phosphorylated forms of GST-Dlg, confirming both the specificity of these antibodies and that the phosphorylation events actually occurs on these residues.

We then proceeded to investigate the pattern of reactivity of these phospho-specific antibodies with endogenous Dlg during different phases of the cell cycle in HaCaT cells. To do this, cells were either grown asynchronously for comparison, or arrested with Aphidicolin and subsequently released into fresh medium. The cells were then harvested at different time points where the 5 hour time point coincided with the S phase of the cell cycle and the 8-9 hour time points coincided with the M phase. This was confirmed by FACS analysis. The extracts from each time point were subjected to immunoprecipitation of hDlg using the anti-hDlg monoclonal antibody and subjected to SDS-PAGE and western blotting using either the anti-hDlg rabbit polyclonal antibody to detect total hDlg or the antiphospho specific antibodies to detect hDlg phosphorylated on serine 158 or serine 442. The results obtained are shown in Figure 29a and demonstrate that the anti-Dlg polyclonal antibody shows a

fairly constant level of reactivity against Dlg throughout the cell cycle. In contrast, the phospho-specific antibodies show a clear preference for Dlg reactivity during the S phase and the time points leading to the M phase, compared with the much weaker signals in asynchronously growing cells and in early G1. In another approach to verify that Dlg is phosphorylated by the CDKs in vivo, we treated asynchronously growing HaCaT cells with the inhibitor Roscovitine and the extracts were processed as mentioned above. As can be seen from figure 29b, little or no Dlg phosphorylated on serine 158 is seen on treatment with roscovitine confirming that Dlg is a substrate for the CDKs in vivo. Taken together these results demonstrate that Dlg is phosphorylated by the CDKs during different phases of the cell cycle, the consequences of which have potentially profound implications for Dlg localisation and stability.

hDlg phosphorylated on Serine 158 and Serine 442 is nuclear

Having confirmed that hDlg is differentially phosphorylated on serine 158 and serine 442 during different phases of the cell cycle, we sought to further examine the data suggesting that Dlg is excluded from the nucleus where it is not phosphorylated on S158 and S442 (see Figure 25). To do this, we decided to use the new phospho-specific antibodies in combination with a biochemical fractionation assay. HaCaT cells were plated on 10cm culture dishes and grown overnight. The cells were then washed extensively in PBS and fractionated into the nuclear and cytoplasmic fractions. The extracts were then resolved by SDS-PAGE and subjected to western blotting. The integrity of the differential extraction procedure was verified by using the specific nuclear and cytoplasmic markers p84 (a nuclear matrix protein marker) and α-tubulin, respectively. As can be seen from figure 30, hDlg is detected in approximately equal amounts in both the nuclear and cytoplasmic extracts with the rabbit polyclonal anti-hDlg antibody. In contrast, the anti phospho-specific antibodies against serine 158 and serine 442 detect hDlg mainly in the nuclear



Serine 158 : shshi(S)pikpte

Serine 442 : lgqtpa(S)parysp

→ **Eurogentec Inc.**

Figure 27: Peptide sequences containing serine 158 and serine 442 were sent to Eurogentec Inc. for the production of phospho-specific antibodies

Figure 28a

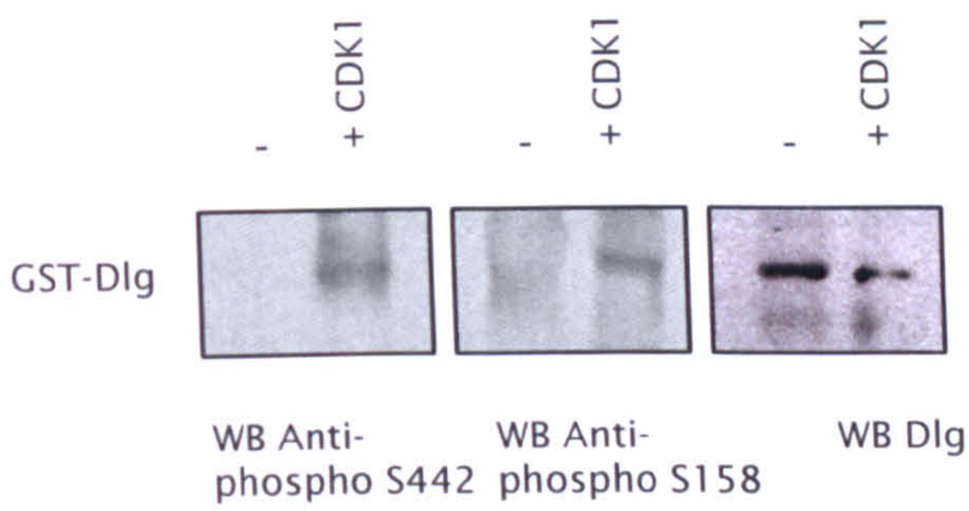


Figure 28b

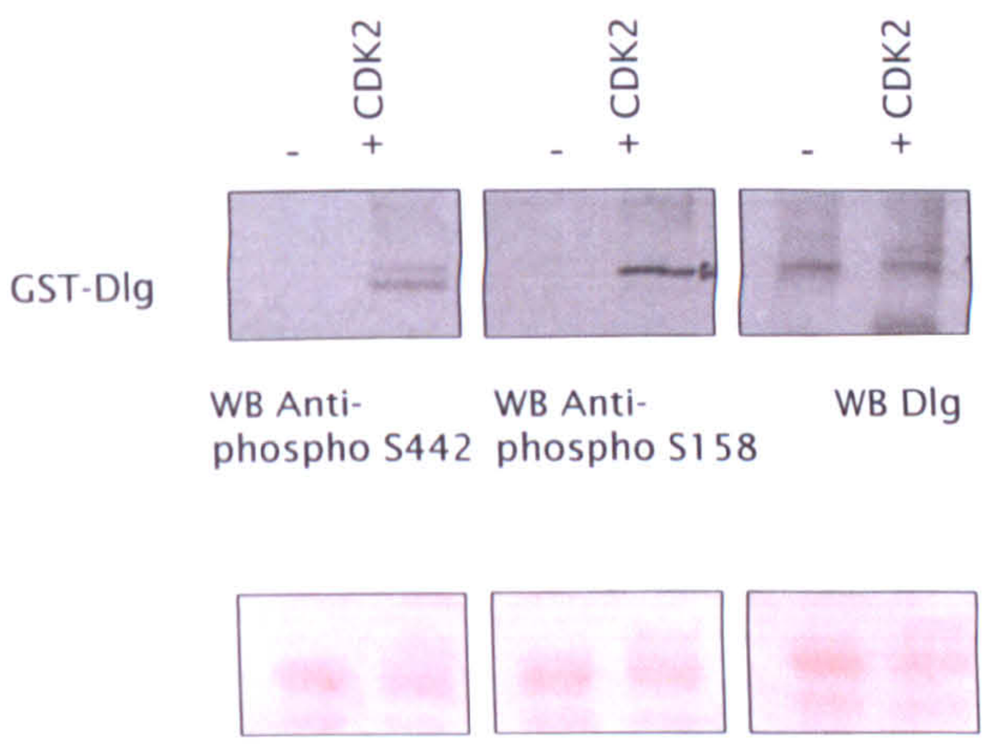


Figure 28 : Dlg phospho-specific antibodies specifically recognise Dlg phosphorylated by cdk1 and cdk2

a) GST-Dlg was either phosphorylated (+) or not (-) in vitro by cdk2 (panel a) and cdk1 (panel b), and then subjected to western blot analysis with anti-Dlg monoclonal antibody (WB Dlg), the anti-phospho-S442 and anti-phospho-158 antibodies. The lower panel shows the ponceau stain of the nitrocellulose membrane used for the cdk1 analysis and demonstrates equal levels of protein loading in all tracks. Note equal levels of reactivity with the anti-Dlg antibody and specific recognition of the phosphorylated forms of Dlg by the anti-phospho antibodies.

Figure 29a

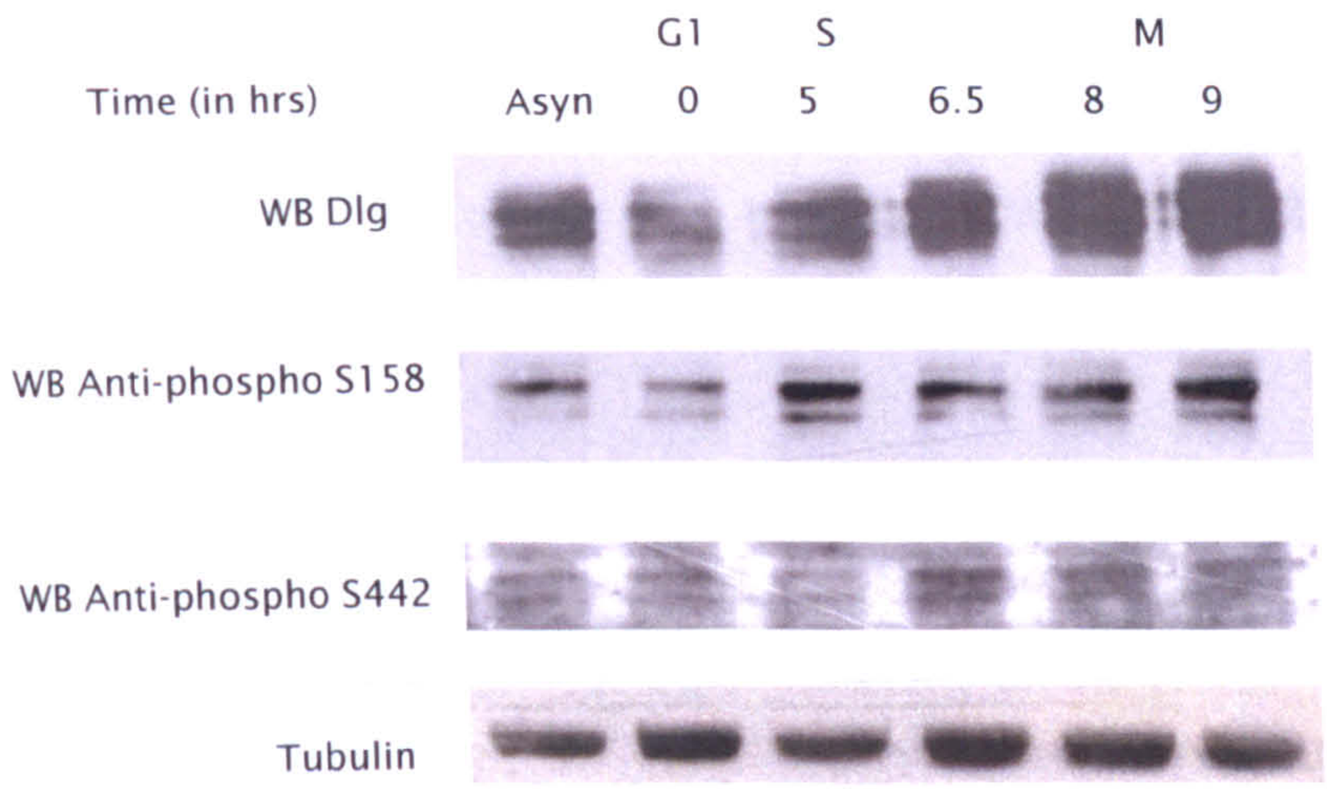


Figure 29b

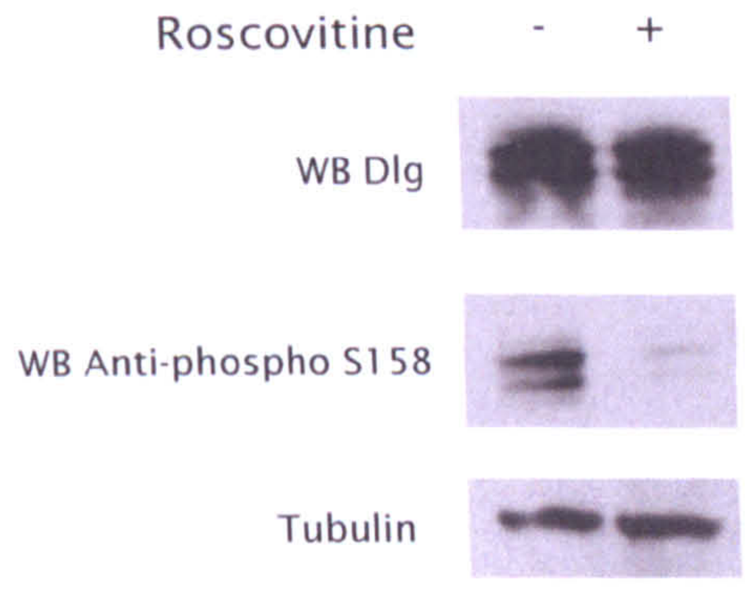


Figure 29:

a) HaCaT cells were synchronised with aphidicolin and then released and cell extracts harvested at different phases of the cell cycle. Dlg was then immunoprecipitated with anti-Dlg monoclonal antibody, and the pattern of Dlg expression analysed by western blotting with anti-Dlg polyclonal antibody (WB Dlg) , the anti phospho-S158 antibody and the anti phospho- S442 antibody. Cell extracts were also analysed using anti-tubulin antibody to confirm equal amounts of protein extraction. Note low level of reactivity of the anti-phospho antibody in asynchronous (Asyn) and G1 arrested cells and the specific increases in S and M phases of the cell cycle.

b) HaCaT cells were either left untreated (-) or treated (+) with 50μM Roscovitine for three hours and then subjected to immunoprecipitation as described above, SDS PAGE and western blotting. The blots were probed for total Dlg and for Dlg phosphorylated at serine 158.

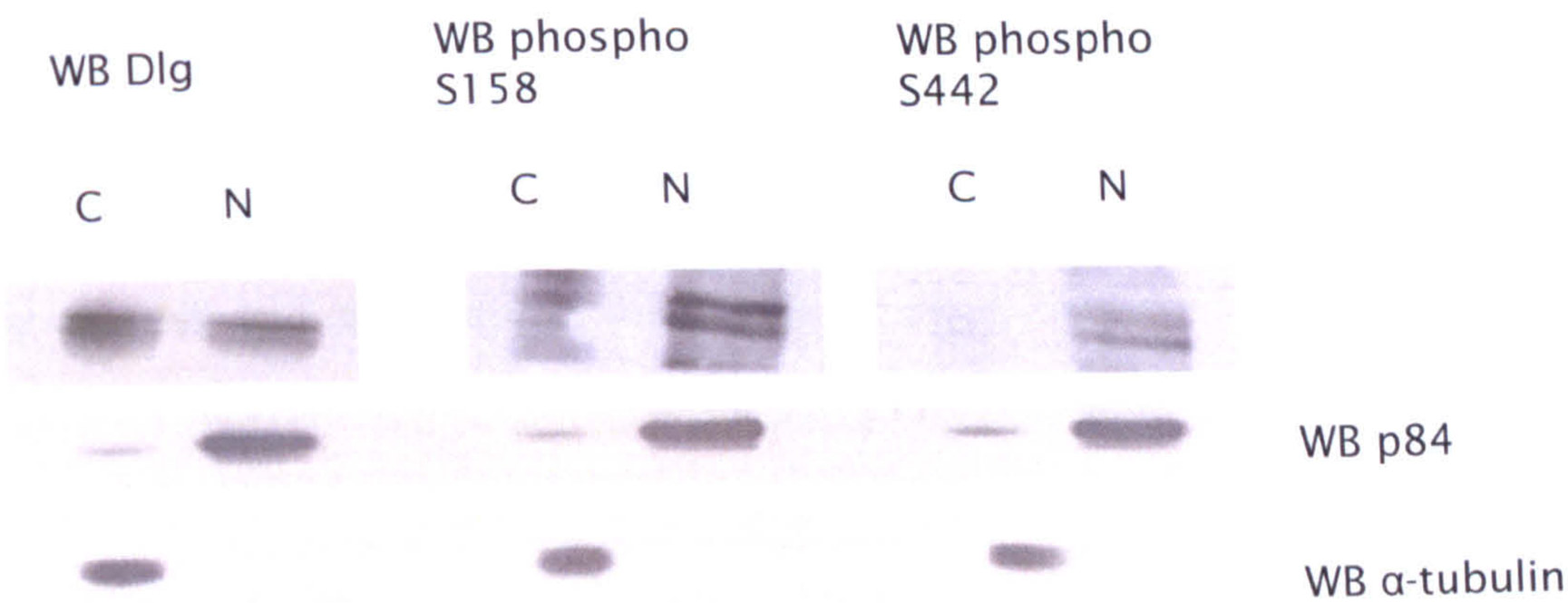


Figure 30 : Nuclear Fractionation of HaCaT cells highlights phosphorylated serine 158 and serine 442 primarily in the nucleus

HaCaT cells were plated on 10cm culture dishes and grown overnight. The cells were then fractionated into the cytoplasmic and nuclear fractions and subjected to western blotting. The blots were probed with the anti Dlg polyclonal rabbit antibody (santacruz) and the phosphoserine 158 and 442 antibodies. P84, a *nuclear matrix* marker and α-tubulin serve as controls for the fractionation experiment.

fraction. This is in concordance with the data obtained from the immunofluorescence analyses of the pattern of expression of the Dlg phospho-mutants (figures 25 b and c), and implies that hDlg phosphorylated on serine 158 and 442 resides largely in the nucleus.

HPV18 E6 and HPV 16 E6 preferentially target nuclear forms of hDlg for proteasome mediated degradation

Previous studies (Massimi et al., 2006) have shown that nuclear forms of hDlg are preferentially degraded by HPV E6. In addition, certain phosphorylation events induced by the JNK and p38 kinases enhanced E6 mediated degradation. Since some of these phosphorylation events occur on serine 158 and serine 442, we were obviously interested in investigating whether the enhanced targeting of phosphorylated and nuclear forms of hDlg by E6 was in any way linked. To address this question, we performed a similar fractionation experiment as the one mentioned above, this time analysing the HPV16-positive cell line CaSKi and the HPV18-positive cell line HeLa. Figure 31a clearly shows approximately equal amounts of hDlg protein in both nuclear and cytoplasmic fractions in HaCaT cells. In contrast both CaSKi and HeLa cells have virtually no hDlg present in the nuclear fraction, whilst there is readily detected cytoplasmic hDlg in CaSki cells. It is known that hDlg is a better target for HPV-18 E6 (Thomas et al., 2001) than for HPV-16 E6, and this reflects in the low levels of hDlg protein seen in the HeLa cytoplasmic fractions of figure 31a. To address whether this lack of nuclear Dlg in CaSKi and HeLa cells was indeed due to proteasome mediated degradation of hDlg, we performed the same experiment, but this time did it in the presence and absence of the proteasome inhibitor MG-132 (figure 31b). As can be seen MG-132 treatment results in a strong increase in the level of hDlg expression in HaCaT, CaSki as well as in HeLa cells. In HaCaT cells, proteasome protection leads to a proportionate increase in hDlg levels in both the nuclear as well as the cytoplasmic fractions.

However, what is striking about CaSki and HeLa cells is the conspicuous restoration of hDlg in the nuclear fraction of both cell lines. It is also interesting to note, that in both cases, the rescue of the protein in the nucleus is predominantly the slower migrating forms of the protein as compared to the cytoplasmic fraction, pointing towards the rescue of the phosphorylated form of hDlg. To confirm this, we repeated the experiment and probed the blots using the phospho-serine specific antibodies. Unfortunately, we were unable to detect phospho-Dlg in HeLa cells, which is most likely due to the very low levels of total hDlg in these cells. However, as can be seen in Figure 31c, the anti phospho-serine 158 antibody recognises hDlg phosphorylated at serine 158 only in the nuclear fraction of proteasome protected CaSki cells. The results indicate that E6 selectively targets the nuclear fraction of hDlg phosphorylated on serine 158, and this is in agreement with previous data from the in vivo degradation assays using the different Dlg mutants (figure 14).

Figure 31a

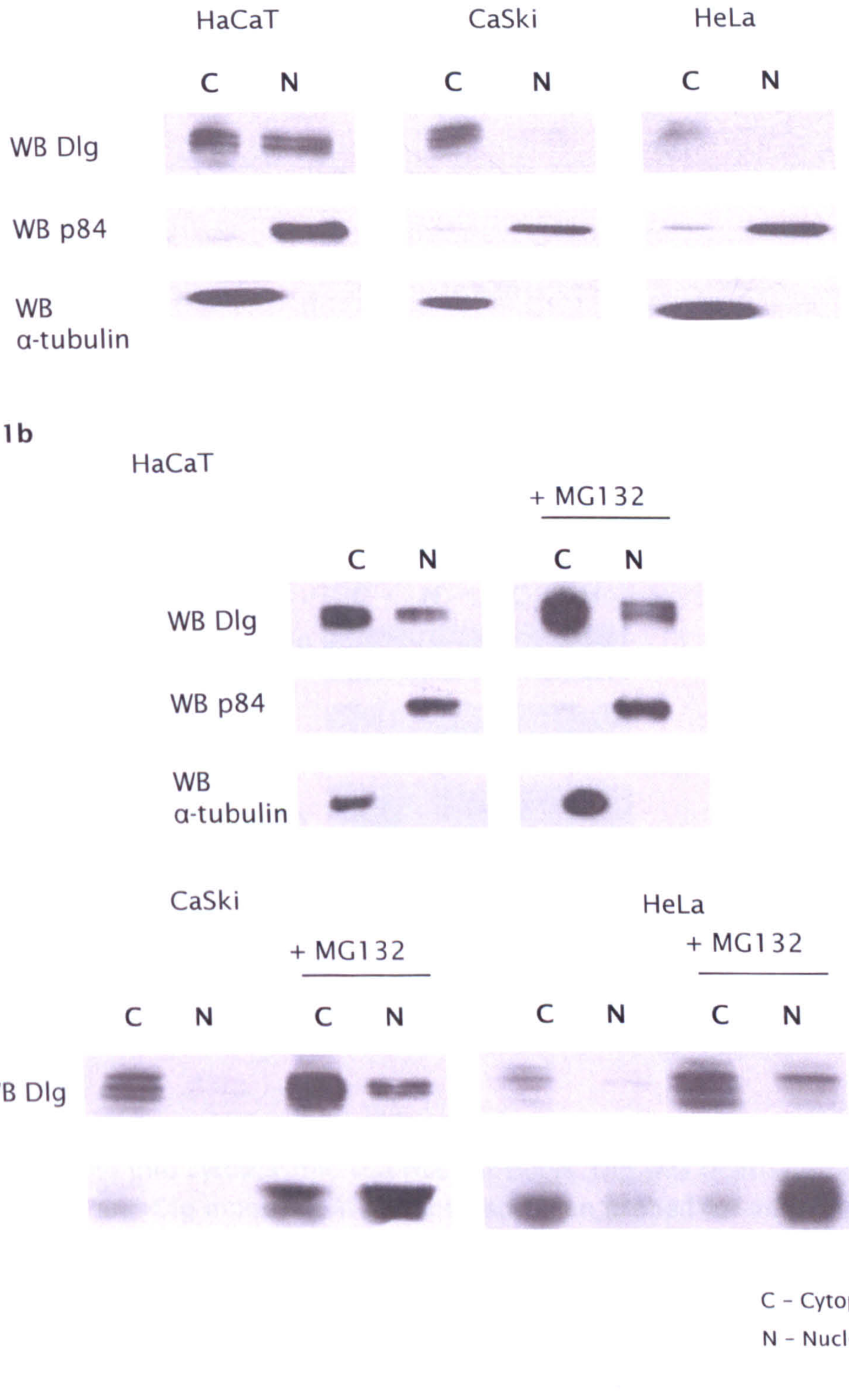


Figure 31b

C – Cytoplasmic Fraction

N – Nuclear Fraction

Figure 31: The nuclear form of hDlg is preferentially degraded by HPV 18 and 16 E6

a) HaCaT, CaSki and HeLa cells were plated onto 10cm culture dishes and processed in the same way as mentioned in figure 23, to yield cytoplasmic and nuclear fractions. The fractions were then subjected to western blotting and the blots probed with an anti-hDlg antibody. b) A similar experiment was performed with these cells with or without the proteasome inhibitor MG-132. p53 is used as a control for the rescue of protein with MG-132. c) CaSki cells were left untreated or treated with MG-132 and then fractionated into cytoplasmic and nuclear pools. Dlg was immunoprecipitated using the anti-Dlg monoclonal antibody and then probed for antiphospho-serine 158.

Figure 31c

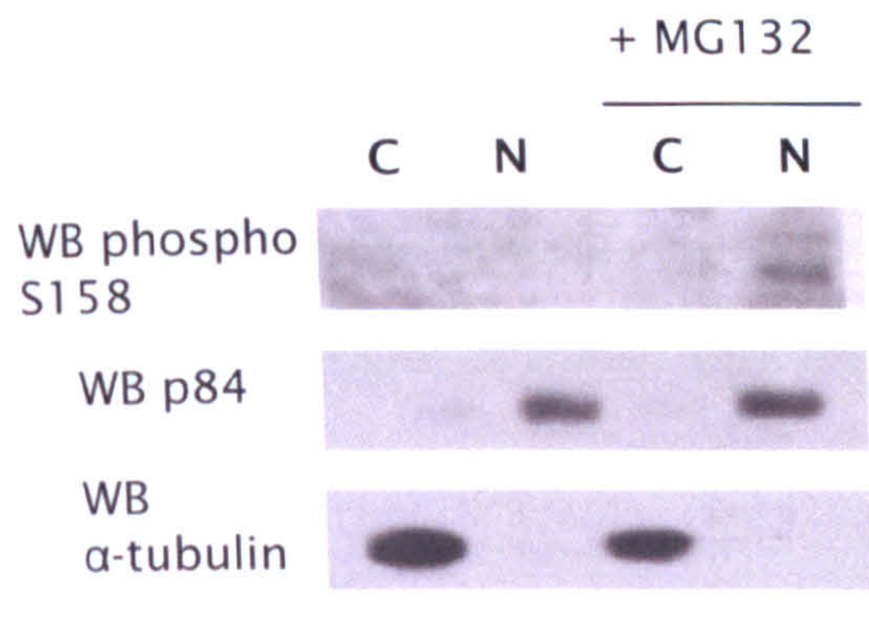


Figure 31 The nuclear form of hDlg is preferentially degraded by HPV 18 and HPV16 E6

c) CaSki cells were left untreated or treated with MG-132 and then fractionated into cytoplasmic and nuclear pools. Dlg was immunoprecipitated using the anti-Dlg monoclonal antibody and then probed for antiphospho-serine 158.

Discussion

Cancer in humans is a multi-step process and each step involves the disruption of a different regulatory circuit within the cell to contribute to the transformation of normal cells into malignant forms (Hanahan and Weinberg, 2000). Among the various steps, perturbations in signal transduction pathways are well recognised as an aid to the progression of neoplastic disease. Signal transduction pathways in cells are a massive array of protein networks that comprise a wide range of molecules, from hormones, cytokines and growth factors to oncoproteins, tumour suppressors and protein kinases. Given the complex nature of these networks, it is easy to see how even a minor misplaced event can trigger cascades that have a catastrophic outcome for the life of a cell.

The main performers in cancer, the tumour suppressor proteins and the oncoproteins, are integral to many of these networks, functioning as important regulators of these networks, and themselves also being subjected to regulatory modifications. One such common modification is phosphorylation, which is an important mechanism of protein regulation that has a major role in practically all aspects of cellular biology. Phosphorylation of tumour suppressors can have diverse effects depending on the particular context. In the case of pRb phosphorylation and its subsequent inactivation in some sporadic cancers, can help these cells to survive (Chau and Wang, 2003). Alternatively, in the case of p53, phosphorylations on specific residues are important for activating the protein by increasing its stability and allowing it to be a delicate cellular response to stress (Steegenga et al., 1996).

A role for Dlg has been established in the regulation of cell polarity, adhesion and junction stability (Abbott et al., 1992, Woods et al., 1996, Humbert et al., 2003), as a controller of development (Caruana et al., 2001, Iizuko-kago et al., 2007) as well as in synapse regulation at both the mammalian and the Drosophila neuromuscular junction (Sanford et al., 2004, Lahey et al., 1994, Zhang et al., 2007, Mendoza-Topaz et al., 2008). However, the actual role of mammalian Dlg in the control of cell growth and cell polarity has been a matter of much speculation. This is complicated by the fact that the molecular mechanisms regulating Dlg function, stability and localisation are still largely unknown. However, similar to many critical regulators of cellular homeostasis, it seems that phospho-regulation of Dlg is going to represent a major aspect of Dlg function. We already know that Dlg is phosphorylated by several kinases. In addition, analysis of the hDlg amino acid composition shows that 14% of the protein is comprised of serine and threonine residues (figure 32). Previous studies have indeed suggested that phosphorylation was likely to play a critical role in the functioning of the mammalian Dlg protein (Mantovani and Banks 2003; Massimi et al., 2006). In this study, the role of two major groups of cellular kinases, the MAPKs (Mitogen Activated Protein Kinases) and the CDKs (Cyclin Dependent Kinases) have been analysed with respect to the regulation of the Dlg tumour suppressor.

hDlg as a substrate for JNK and p38MAPK

hDlg localisation to the membrane and the endosomes

A particularly interesting observation from these studies is the demonstration that Dlg accumulates at sites of cell contact and in numerous vesicular structures in the cytoplasm following exposure to sorbitol induced osmotic stress. These were confirmed using specific endosomal markers to which hDlg was found to colocalise with following osmotic stress. It is worth noting that

1 MPVRKQDTQRALHLLEEYRSKLSQTEDRQLRSSIERVINIFQSNLFQALIDIQEFYEVTL

61 LDNPKCIDRSKPSEPIQPVNTWEISSLPSSTVTSETLPSSLSPSVEKYRYQDEDTPPQEH

121 ISPQITNEVIGPELVHVSEKNLSEIENVHGFVSHSHISPIKPTEAVLPSPPTVPVIPVLP

181 VPAENTVILPTIPQANPPPVLVNTDSLETPTYVNGTDADYEYEEITLERGNSGLGFSIAG

241 GTDNPHIGDDSSIFITKIITGGAAAQDGRLRVNDCILQVNEVDVRDVTHSKAVEALKEAG

301 SIVRLYVKRRKPVSEKIMEIKLIKGPKGLGFSIAGGVGNQHIPGDNSIYVTKIIEGGAAH

361 KDGKLQIGDKLLAVNNVCLEEVTHEEAVTALKNTSDFVYLKVAKPTSMYMNDGYAPPDIT

421 NSSSQPVDNHVSPSSFLGQTPASPARYSPVSKAVLGDDEITREPRKVVLHRGSTGLGFNI

481 VGGEDGEGIFISFILAGGPADLSGELRKGDRIISVNSVDLRAASHEQAAAALKNAGQAVT

541 IVAQYRPEEYSRFEAKIHDLREQMMNSSISSGSGSLRTSQKRSLYVRALFDYDKTKDSGL

601 PSQGLNFKFGDILHVINASDDEWWQARQVTPDGESDEVGVIPSKRRVEKKERARLKTVKF

661 NSKTRDKGEIPDDMGSKGLKHVTSNASDSESSYRGQEEYVLSYEPVNQQEVNYTRPVIIL

721 GPMKDRINDDLISEFPDKFGSCVPHTTRPKRDYEVDGRDYHFVTSREQMEKDIQEHKFIE

781 AGQYNNHLYGTSVQSVREVAGKGKHCILDVSGNAIKRLQIAQLYPISIFIKPKSMENIME

841 MNKRLTEEQARKTFERAMKLEQEFTEHFTAIVQGDTLEDIYNQVKQIIEEQSGSYIWVPAKEKL

Figure 32: The amino acid sequence of hDlg taken from http://www.ncbi.nlm.nih.gov/sites/entrez (Disks large homolog 1 (Synapse-associated protein 97) (SAP-97) Accession number: Q12959) showing the serines highlighted in red and the threonines in blue.

the marker Transferrin is found both in early endosomes as well as in recycling endosomes. The only way to address the specificity of hDlg to one of the two pathways (or both) is to subject the protein to a live imaging assay and follow the movement hDlg within these vesicle-like structures. In addition, treatment of cells with sodium monensin, a potent inhibitor of endosomal pathways, generally decreased both the accumulation of hDlg at sites of cell contact and its appearance in these vesicular structures following osmotic stress. It is evident that the maintenance of cell polarity in epithelial cells largely depends on the trafficking of apical and basolateral cargoes to their respective locations, and that this trafficking of proteins is largely an endosomal vesicle-mediated process (Folsch 2007; Lu and Bilder 2005). In Drosophila, polarity defects induced by mutations in two components of endocytic vesicle machinery - namely Syntaxin and Rab - were accompanied with over-proliferation of tissue (Lu and Bilder, 2005). Interestingly, a similar pattern of increased membrane staining and accumulation in these vesicular structures was also observed with the other two components of the Scrib polarity complex, hScrib and Hugl-1 (Massimi et al., in press). This highlights the fundamental importance of the link between proteins that form the polarity complexes at the cellular junctions and their transport by endocytic vesicles. Therefore our data is the first demonstration of an alternative trafficking route for the hScrib mammalian polarity complex. In agreement with the work of Lu et al., we also observe an association of each of the components of the hScrib polarity complex, hDlg, hScrib and Hugl-1, with one another and with the tSNARE Syntaxin4, and these interactions are enhanced under conditions of osmotic stress (Massimi et al., in press). It is quite possible that the induction of osmotic shock might actually be interfering with the dynamic transport of the endosomes to and from the membrane, resulting in their apparent static accumulation within the cytoplasm along with the Scrib complex, giving rise to these vesicle-protein aggregates as is consistently seen in the

immunofluorescence assays. The discovery of this new route to a familiar place for hDlg and the other components of the hScrib polarity complex, confirms in mammalian cells the established link between polarity proteins and endocytic transport pathways (Lu and Bilder 2005; Balklava et al., 2007; Leibfried et al., 2007).

The disruption of the relocalisation of hDlg to the membrane and to these vesicles by inhibiting JNK suggests that the putative phosphorylation of hDlg by JNK is a prerequisite for this stress induced relocalisation to occur. The JNK kinase has been previously shown to function in planar cell polarity and the regulation of epithelial morphogenesis (Noselli and Agnes, 1999), and is activated and acts as a signal transducer in the Wnt pathway (Pandur et al., 2002). Intriguingly, hScrib and Hugl-1 also depend on JNK for their relocalisation to the membrane following osmotic shock (Massimi P., in press), suggesting that phosphorylation of the hScrib polarity complex maybe a mechanism by which the component proteins act in concert at the cell membrane. Also, as shown by Massimi et al (2006), relocalisation appears to require an active transport mechanism, most likely involving actin filaments, since the movement of Dlg was dramatically reduced following exposure of the cells to cytocholasin B which is an inhibitor of the polymerisation of actin filaments. Though the formation of the endocytic vesicles might be independent of JNK activity, the localisation of hDlg to the vesicles, and consequently to the membrane, seems to be regulated by hDlg's phosphorylation by JNK (Figure 33). hScrib and Hugl-1 also appear to be regulated in an analogous manner, however it remains to be determined whether either protein is indeed a substrate for JNK. Both proteins do contain numerous potential consensus sites for phosphorylation by the MAPKs.

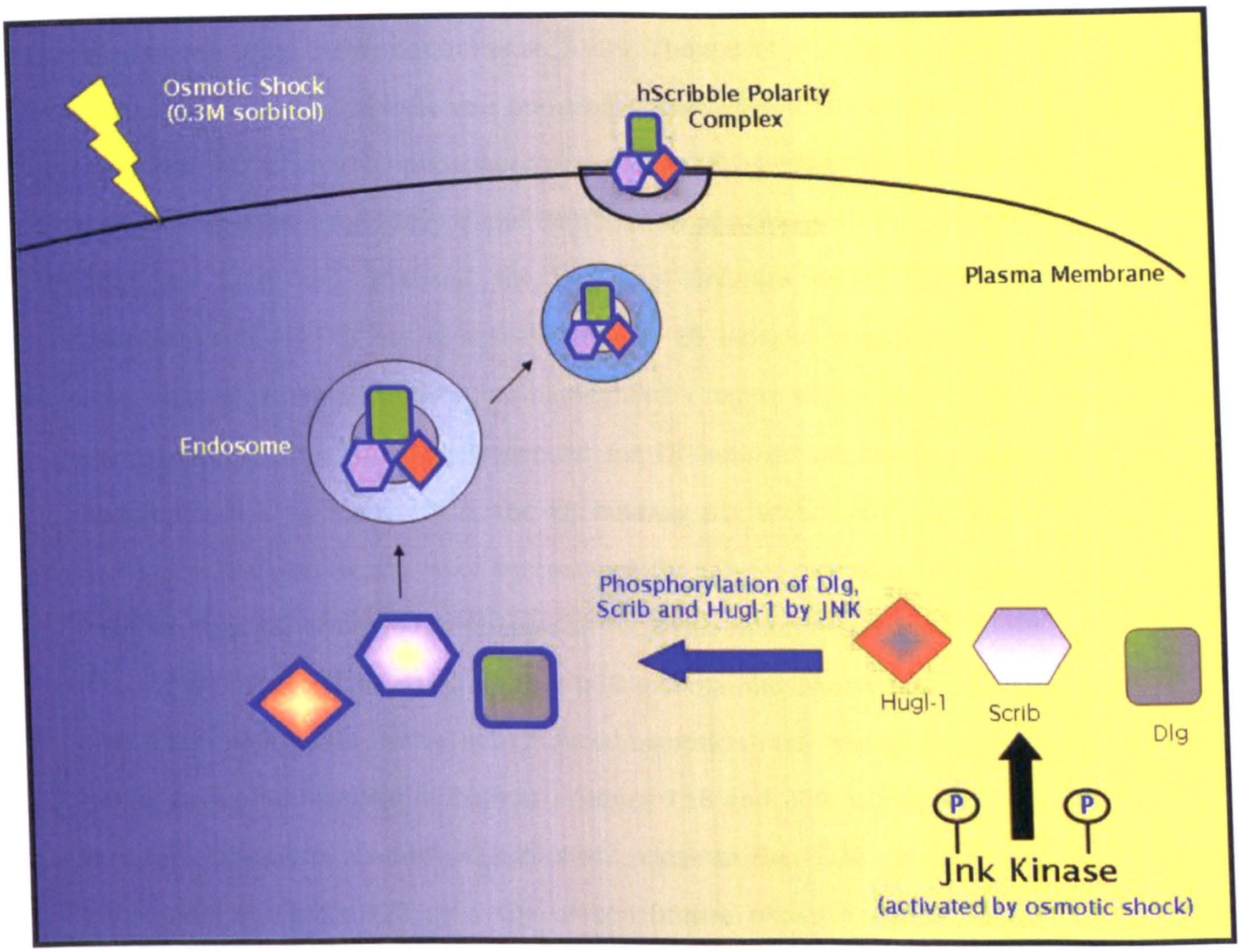


Figure 33 : An illustration showing the change in localisation of the hScrib polarity complex consisting of hDlg, hScrib and Hugl-1 on Osmotic Shock and consequent JNK phosphorylation.

Osmotic shock induced phosphorylation of hDlg and susceptibility to HPV E6

The degradation of hDlg by HPVE6 is a well established and documented event (Kiyono et al., 1997; Gardiol et al., 1999; Thomas et al., 2001). Previous studies have demonstrated the role phosphorylation of HPV E6 can play in E6 targeting of hDlg, whereby phosphorylation of the PDZ binding site of HPV18 E6 by PKA abrogates the binding and degradation of hDlg by it (Kuhne et al., 2000). We have now analysed the converse situation where effects of phosphorylation of Dlg on its susceptibility to E6 induced degradation has been analysed. Previous studies had highlighted a region within the first 185 amino acids of hDlg as being important for E6-induced degradation, even though this lies well away from the E6 binding site within PDZ domain 2, suggesting that this region may be required for efficient binding of cellular ubiquitin ligase(s) or other components of the degradation machinery (Gardiol *et al.*, 2002). Sabio et al identified four p38 gamma phosphorylation sites on hDlg following exposure to sorbitol induced osmotic stress; two of these were within the amino terminal domain at residues 158 and 209, whilst the other two were located at residues 431 and 442, close to the PDZ2 domain. They also identified Serine 122 as a site of constitutive phosphorylation of Dlg. Interestingly, in our analysis, we saw no significant increase in the migration of the ΔNT truncation of Dlg (which lacks serine 122 and serine 158), suggesting that phosphorylation of Dlg after osmotic shock may be cooperative. Indeed, we show that mutating serine 158 to an alanine itself is enough to account for a massive mobility shift in the protein. However the ΔNT mutation was nonetheless constitutively phosphorylated; the identity of this constitutively phosphorylated residue was suggested to be Serine 122 by Sabio et al. This implies Serine 158 as the major site required for enhanced E6 targeting. Indeed using Dlg mutants that are defective for phosphorylation at residues

158 and 209 confirmed this, with 209 behaving like wild type Dlg and 158 not being subject to increased degradation following osmotic shock.

Although this tells us that phosphorylation of hDlg in the amino terminal region, specifically at serine 158, enhances E6 mediated degradation, the complicated dual kinase regulation of hDlg by both p38MAPK and JNK makes it difficult to pinpoint the exact event that makes this possible. The work of Sabio et al proposes that the osmotic shock induced phosphorylation of hDlg releases it from its bound state to the protein GKAP, and thereby releasing it from the cytoskeleton. However, this does not seem to be substantiated by our immunofluorescence and biochemical fractionation data. In both cases we observed a strong accumulation of Dlg at the cell membrane, and this is accompanied by an equivalent increase in the insoluble cytoskeletal bound fraction of the protein. Technically, it was possible that this apparent relocalisation of hDlg was a reflection of enhanced degradation of the protein in the soluble fraction, however we can exclude this since proteasome inhibition did not rescue proportional amounts of Dlg in either fraction. It therefore seems most likely that the reason for the apparent differences between the two studies is due to cell line dissimilarities. It is worth emphasising here that we mainly used endogenous hDlg from HaCaTs, which are human skin keratinocytes whilst Sabio et al used an overexpression system in HEK293 cells and endogenous Dlg from mouse embryo fibroblasts. Putting the osmotic shock induced phosphorylation of hDlg in perspective with its interaction with HPV E6, we can conjecture that following phosphorylation, Dlg is released and begins its journey towards the cell membrane; en route it is accessible to the HPV E6 protein and is consequently targeted for proteasome mediated degradation. Once at the membrane, it seems likely that hDlg's function is to aid the cell in responding to changes in the extracellular environment by participating in multi-protein complexes whose functions

probably contribute to maintaining cell polarity, membrane integrity and cell-cell contact.

hDlg as a substrate for the CDKs

Given the unknowns in the functioning of mammalian Dlg as a growth regulator, we thought a good way to start examining its actual role in this context would be to observe its behaviour during the cell cycle. hDlg has certainly been implicated in a cell cycle regulatory function through its association with the APC tumour suppressor (Ishidate et al., (2000), and the mitotic PDZ binding kinase (Gaudet et al., 2000). Recent studies have also suggested that different isoforms under certain circumstances may have different effects upon cell proliferation through activation of the PI-3 kinase pathway (Frese et al., 2006). This may in fact be linked to reported differences in the cellular localisation of different Dlg isoforms (McLaughlin et al., 2002; Roberts et al., 2008). It now seems likely that many of these diverse activities and patterns of expression may actually be connected through common pathways of post-translational modification of Dlg.

Therefore we first proceeded to analyse the pattern of hDlg expression in terms of localisation as well as to characterise it biochemically during different phases of the cell cycle. Analysis of asynchronously growing HaCaT cells reveals a range of different Dlg patterns of expression, including diffuse cytoplasmic localisation, and that to the mitotic spindle and the midbody, suggesting a degree of cell cycle regulation. When HaCaT cells were arrested in aphidicolin and Dlg expression then analysed upon release, relocalisation of Dlg was also observed during different phases of the cell cycle, varying from membrane bound/diffuse patterns of expression, to an enhanced degree of cytoplasmic expression during S phase and accumulation on the mitotic spindle during mitosis. There is also a striking accumulation of hDlg at the

midbody which has also been reported previously (Massimi et al., 2003). Although we definitively show the change in hDlg localisation along with the progression of the cell cycle as well as the fact that it is phosphorylated differentially at different stages, western blot analysis of Dlg expression during the cell cycle strongly suggests the presence of differentially modified forms of the protein during different phases of the cell cycle. The Cyclin Dependent Kinases (CDKs) were obvious candidate kinases given their tight involvement in regulating the cell cycle as well as the presence of corresponding consensus sites on the hDlg sequence. It is quite possible that the progressive change in localisation during the cell cycle and the posttranslational modification of hDlg are two entirely independent events, and the data from Massimi et al., (2003) which imply the C terminus as at least being responsible for the midbody accumulation would certainly argue that the CDK phosphorylation of hDlg on two residues that both lie in the N terminal half of the protein, do not have a role to play in this pattern of protein staining. However the conformational change induced by such a phosphorylation event cannot be ruled out and therefore until experimental data unquestionably proves otherwise, it would be difficult to contemplate on this aspect.

The CDKs phosphorylate hDlg on Serine 158 and Serine 442

Indeed, we show that Dlg is a substrate for cdk1 and cdk2 both in vitro and in vivo. This was done firstly using purified components in vitro, and mutational analysis combined with bioinformatics analysis of the hDlg amino acid sequence allowed us to identify that S158 and S442 were both phospho-acceptor sites for cdk1 and cdk2. Multiple experiments provide the data supporting CDK regulation of Dlg in vivo. We observe a co-immunoprecipitation between endogenous Dlg and cdk2, as well as between Dlg and the cdk1 subunit cyclin B, demonstrating that Dlg can at least be found in the complexes that would be expected to have CDK activity. We also

observed a clear difference in the pattern of Dlg migration on SDS PAGE when cells were exposed to the CDK inhibitor, Roscovitine, in both G2/M as well as in asynchronously growing cells, where the treated cells lose the higher migrating phosphorylated forms of the protein, suggesting that CDKs do in fact phosphorylate endogenous Dlg. Finally, we also generated phospho-specific antibodies that show a clear cell cycle dependent phosphorylation of Dlg on S158 and S442.

It is intriguing to note that S158 and S442 were also identified as being sites of phosphorylation by p38gamma following exposure of cells to osmotic stress (Sabio et al., 2005), while our studies show that these sites are also phosphorylated during the course of a normal cell cycle. As a result of these phosphorylation events following osmotic shock, Dlg lost its ability to bind GKAP and the cytoskeleton. It is tempting to speculate that phosphorylation of Dlg during a normal cell cycle by the CDKs at these two sites may also play a similar function and thereby correctly regulate the localization of Dlg during different phases of the cell cycle. It is however, impractical to ignore the interplay between the MAPK and the CDK networks, as it is obvious that although both kinase groups partly phosphorylate the same residues, there is a marked difference in the functions they govern. The location of hDlg upon phosphorylation seems key here, as the MAPK phosphorylation sends it to the membrane, while the CDK phosphorylation locates hDlg to the nucleus (see figure 34). However the p38MAPK, also phosphorylates at least two other residues on hDlg, threonine 209 and serine 431 (Sabio et al., 2005), and the observed result for the MAPK governed phenotype might well be a cumulative effect of these multiple phosphorylation events put together. It would be of great value to identify the cellular binding partners of Dlg whose interactions may be modified as a consequence of these phosphorylation events, and thereby gain a better understanding of the true significance of its

phosphorylation in terms of interacting networks in the cell. It is remarkable to note that serine 442 lies between the PDZ2 and PDZ3 domains of the protein, which is an area involved in binding several proteins involved in the regulation of cell proliferation. These include the APC tumour suppressor protein (Matsumine et al., 1996), and cellular oncoproteins such as Net1. In addition, viral oncoproteins such as HPV E6, 9ORF1 and HTLV Tax1 (Kiyono et al., 1997) also bind to PDZ2. Obviously an intriguing question to pursue will be to determine whether any of the Dlg interaction is in any way regulated by the S442 phosphorylation event.

CDK Phosphorylation and the Stability of hDlg

One of the important consequences of the CDK phosphorylation event seems to be a major structural alteration in the Dlg protein. Dlg normally migrates as two major species and interestingly, the S158D mutation is alone sufficient to generate the slower migrating form of the protein, suggesting that the presence of an acidic residue at this position induces a major structural change in Dlg. Given the probable change in structure as well as the alteration in phosphorylation status, it seemed correct to consider a possible link between these events and changes in protein stability. Interestingly, phosphorylation at these sites also appears to regulate the level to which Dlg is ubiquitinated. Certainly, the lack of acidic residues at S158 and S442 greatly shortens the half-life of the protein, whilst in contrast; the presence of two acidic residues significantly extends the half-life and produces a dramatic decrease in the level of ubiquitination. Obviously these assays were performed with ectopically expressed mutants and it will now be interesting to determine whether different species of Dlg have different levels of stability during different phases of the cell cycle. However, indications are that there are differences in the levels of Dlg expression through the cell cycle and blocking

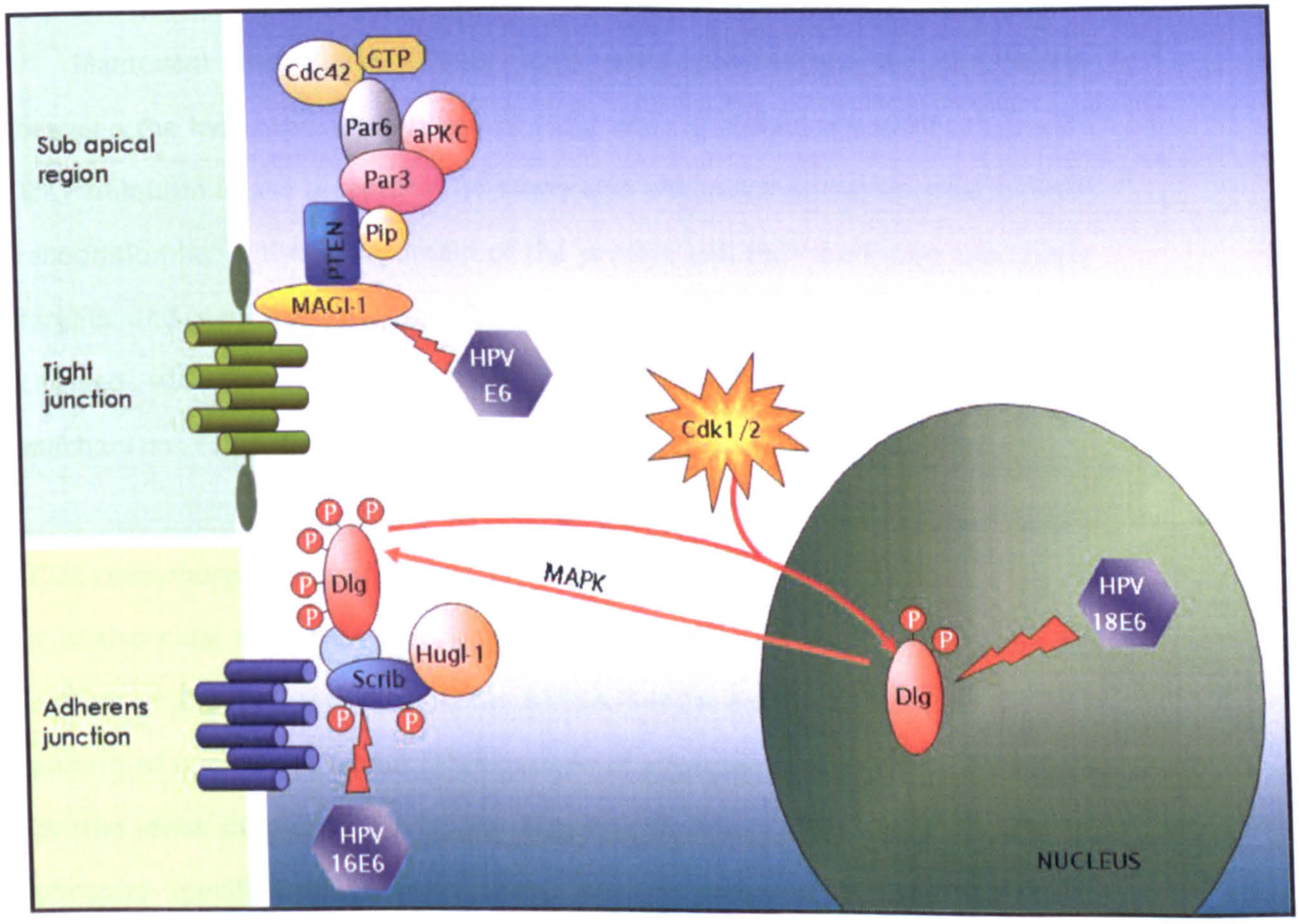


Figure 34 : An illustration depicting the regulation of Dlg by the MAPKs and the CDKs (see text for details)

CDK activity with roscovitine treatment reduces the level of Dlg, which is consistent with the data with the ectopically expressed mutants.

Mantovani and Banks (2003) have previously shown an association between the hyperphosphorylation of hDlg and its affinity thereafter to the β-TrCP ubiquitin ligase receptor. The study also showed that the sites for β-TrCP recognition lay in the SH3 domain of the protein and that the ligase selectively targets and degrades the membrane-cytoplasmic pool of hDlg. This data coupled with our study strongly suggest the involvement of multiple mechanisms regulating the stability of the hDlg in a location based manner.

CDK phosphorylation and the nuclear localisation of hDlg

It is also clear that phosphorylation at S158 and S442 most likely alters the pattern of Dlg expression, with the S158A/S442A double mutant exhibiting a pattern of nuclear exclusion. This pattern of expression was supported further by the data obtained in cellular sub-fractionation experiments using the phospho-specific antibodies, where phosphoserine 158 and 442 were detected in the nucleus of HaCaT cells. There is little work detailing the presence of hDlg in the nucleus or why it should be found there. The earliest reports from McLaughlin et al (2002) proposed that nuclear hDlg is isoform-specific and only the I2 isoform localises to the nucleus while the I3 isoform stays excluded. The locations of the two serines exclude any sites of alternative splicing and are a common attribute to all different isoforms of hDlg and hence we can confidently state that we show a localisation to the nucleus based on a posttranslational modification only. Massimi et al (2004) demonstrated that E6 specifically targets nuclear and not membrane-cytoplasmic forms of hDlg. We now show that this is indeed true and that on nuclear fractionation of the high risk HPV genome containing cell lines HeLa and CaSki, very small amounts of hDlg is seen in the nuclear fraction. However

on blocking proteasome mediated degradation of Dlg using the proteasome inhibitor MG-132 prior to the fractionation experiment, a rescue of the nuclear fraction is immediately seen in these two cell types. It is therefore likely that the E6 oncoprotein targets nuclear phosphorylated hDlg to stop it from performing certain special functions in the cell nucleus.

So what might be the possible role of hDlg in the nucleus? The interaction of the Rho exchange factor Net1 with hDlg promotes its localisation to nuclear subdomains associated with PML bodies (Garcia Mata et al., 2007). The N-terminal deleted oncogenic form of Net1 meanwhile sequesters hDlg in the cytosol leading to cellular transformation and providing yet another example of the critical, but as yet undocumented role for hDlg in the nucleus. In keeping with its role as a tumour suppressor, hDlg has been shown to behave as a transcriptional repressor in concert with p300 (Massimi P., personal communication). It remains to be seen if this co-operation requires the phosphorylation and consequent translocation of hDlg to the nucleus.

Combined Observations

The most striking observation to be made from this study is the differential phosphorylation of Serine 158 and its related consequences. We and others (Sabio et al., 2005) definitively show the phosphorylation of this residue under osmotic shock by the p38MAPK. Additionally we also demonstrate that the Serine 158 forms part of a CDK consensus and is phosphorylated also by both CDK1 and CDK2. The drastic change in migration of the protein upon making one single point mutation that changes this serine to an alanine shows that the phosphorylation of this residue might be pivotal in determining the conformation of the protein and also in estimating subsequent downstream phosphorylation events on hDlg. Its phosphorylation

also seems to determine hDlg susceptibility to HPV E6. This may be due to the structural affinity this modification may confer to hDlg, but it may also in part be the change in location. The translocation of hDlg to the nucleus upon phosphorylation by the CDKs might make it more accessible to the E6 oncoprotein which has a largely nuclear localisation, thus improving the efficiency of E6-mediated degradation of the hDlg protein. The evolution of the ability of two different kinases to phosphorylate the same residue - this being true also for serine 442 - highlights the importance of this modification in the cellular function of hDlg.

It is quite likely that single phosphorylation changes such as this might serve as a "master regulator" of hDlg in some cellular contexts. Accordingly, a model that seems particularly attractive in explaining why hDlg has such an astonishing repertoire of responses to changes in phosphorylation might stem from the regulation of the interactions of hDlg with other proteins. Consequently, if changes in phosphorylation modulate interaction with different proteins in different systems, then a basic mechanism for how this occurs would involve hDlg charge. Presumably, changes in the charge would modify protein/protein contact through changes in hDlg conformation caused by the phosphorylation of the protein. This in turn will certainly affect its functional interactions with important binding protein partners, triggering a torrent of changes in cellular networks.

Future work: hDlg as a substrate for other cellular kinases

This study shows the importance of the phosphorylation of hDlg not only in controlling protein stability, function and localisation, but also its relevance to neoplastic disease. The ability of hDlg to be phosphorylated by multiple kinases reflects its involvement in several cellular signaling pathways. Examples of other important cellular players establish that one of the ways in which proteins with the most crucial roles in the cell show their versatility, is by being able to take part and to function in diverse pathways depending on their phosphorylation - p53 is phosphorylated by an assortment of kinases including CKI, ATM, ATR, Chk2, JNK, GSK3-β in response to various cellular stresses such as DNA damage, UV or H_2O_2 treatment and replicative senescence (Lavin and Gueven, 2006); c-myc is phosphorylated by the MAPKs and GSK3-β leading to changes in its transforming capabilities (Henriksson et al., 1993; Vervoorts et al, 2006). The retinoblastoma protein Rb is inactivated upon phosphorylation by CDK4 and CDK6, disrupting its complexes with E2F and allowing entry into S phase of the cell cycle (Chau and Wang, 2003). Given the abundance of residues in hDlg that can be phosphorylated, it was of obvious interest to us to know what other kinases hDlg might be a substrate for. In an attempt to shed more light on the involvement of hDlg in other cellular signaling pathways in the cell, we scanned the amino acid sequence for the consensus sites of numerous kinases that have critical roles in cellular processes. Of the many sites, we found a large number of perfect consensus sites for three kinases known to be involved in the regulation of the Wnt pathway - CKI, CKII and GSK3-β. (Figures 35 and 36). Figure 37 shows an invitro kinase assay where GST-Dlg fusion protein was incubated with kinase

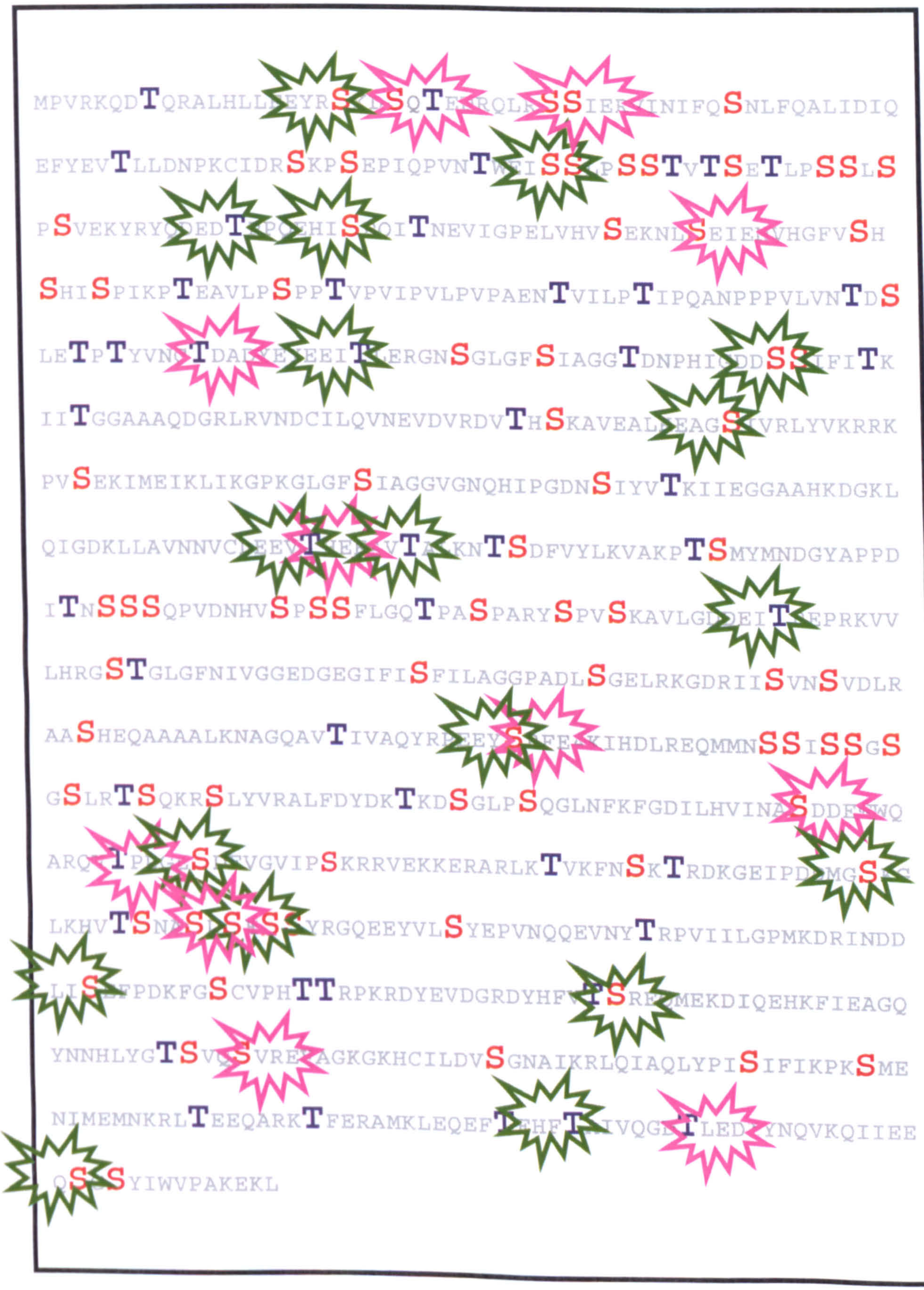


Figure 35: The amino acid sequence of hDlg with highlighted consensus sequences for the kinases CKI (green), CKII (pink)

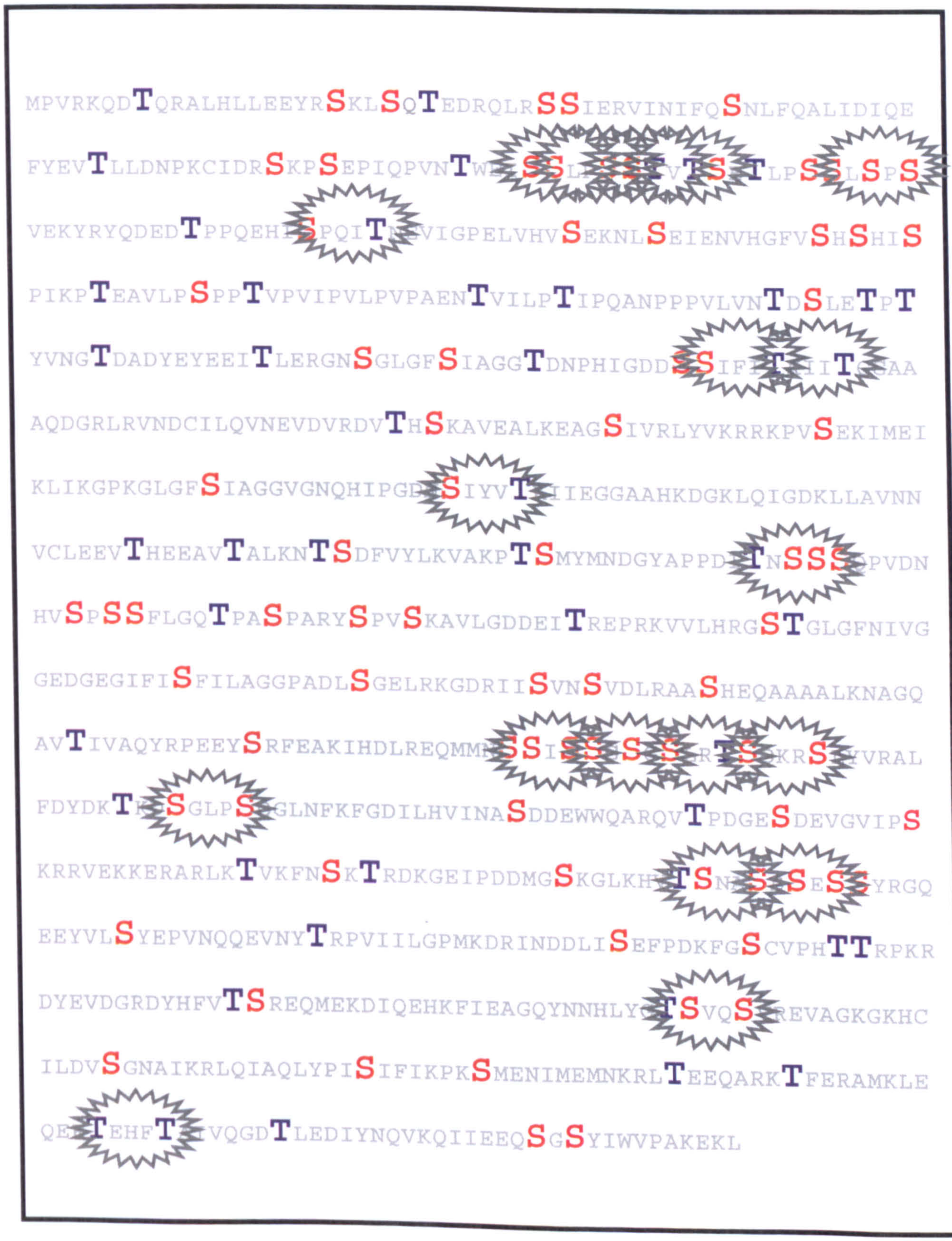


Figure 36: The amino acid sequence of hDlg with highlighted consensus sequences (grey) for the GSK3-β kinase.

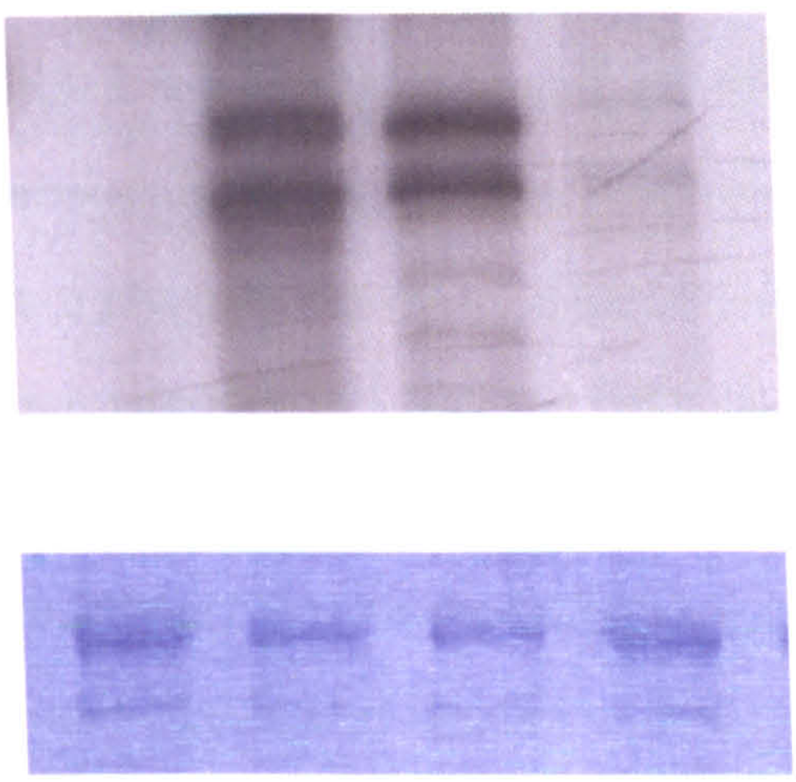


Figure 37: Phosphorylation of Dlg by the kinases CKI, CKII and GSK-3β.

GST-Dlg was incubated with no kinase or purified CKI, CKII and GSK-3β plus radio-labelled ATP for 20min, and phosphorylation of Dlg ascertained by SDS-PAGE and autoradiography (upper panel). The lower panel shows the Coomassie stained gels showing the control GST-Dlg with no enzyme and the GST-Dlg fusion proteins incubated with enzyme.

buffer, radiolabelled ATP and the respective purified kinases. As can be seen, Dlg is a good substrate for CKI and CKII and a moderate one for GSK3- β.

CKI phosphorylates the consensus motif D/E-X-X-S/T and CKII phosphorylates the S/T-X-X-E/D motif (where X is any amino acid). Casein Kinase 1 and Casein Kinase 2 belong to a family of serine/threonine protein kinases, and have both been implicated in the regulation of various cellular functions, including membrane transport, cell division, apoptosis, chromosome segregation and the circadian rhythm (Knippschild et al., 2005; Song et al., 2000). GSK3-β is a serine/threonine protein kinase that binds and phosphorylates the consensus S/T-X-X-X-$S_{(P)}$ /$T_{(P)}$ (where X is any amino acid and the last residue is a phospho-serine or phospho-threonine). It is a kinase that has potent tumour suppressor qualities through its involvement in cell cycle regulation, apoptosis and growth, as well as a key regulator of numerous signaling pathways including those comprising glycogen metabolism and G-protein coupled receptors (Doble and Woodgett, 2003). The common factor to all three kinases, however, is the Wnt pathway.

The Wnt pathway was first discovered in Drosophila and is a complex network of many proteins, most of which are known to be important in cancer, as well as in normal physiological processes in the cell (Lie et al., 2005). The interaction of hDlg with one of the main players in the Wnt pathway - the APC protein - has already been documented (Matsumine et al., 1996; Ishidate et al., 2000). Matsumine et al also stated that though they did not observe a direct interaction between another member of the Wnt pathway, β-catenin, and hDlg, they do observe APC, β-catenin and hDlg in the same complex. It is already known that the association of APC with β-catenin, the sequential phosphorylation of β-catenin by CKI and GSK3-β and its subsequent degradation is a very important cellular turnover process. The disruption of

Materials and Methods

Cells and transfection.

293 (human embryonic kidney), U2OS (human osteosarcoma, HaCaT (Human keratinocyte), CaSki (HPV16 positive cells) and HeLa (HPV18 cells positive cells) were grown in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (100U/ml) and glutamine (300µg/ml). Transfection was carried out using calcium phosphate precipitation as described previously (Matleshewski et al, 1987) or using Lipofectamine2000 (invitrogen) according to the manufacturer's protocol. The TR2 control cell line and the Dlg-shRNA cell lines were a gift from Dr. Paola Massimi. Experiments gauging endogenous levels of Dlg were performed in HaCaT cells, whereas all over-expression experiments were performed in 293 cells and U2OS cells owing to the technical difficulty of obtaining good transfection efficiency in HaCaT cells.

Plasmids.

PCDNA HA-Dlg: The wild type HA-tagged Dlg expression plasmid has been described previously (Gardiol et al 2002). The point mutations to serine 158 and 442 were constructed using PCR driven site directed mutagenesis (GeneTailor - Invitrogen). The primers for mutating serine 158 are as follows: To alanine - Forward primer 5' GTCTCTCACTCTCATATCGCACCCATAAAG; and to aspartic - Forward primer 5' GTCTCTCACTCTCATATCGACCCCATAAAG and the reverse primer 5' GATATGAGAGTGAGAGACAAACCCGTGGAC. The primers for mutating serine 442 are as follows: To alanine - Forward primer 5' TTGGGCCAGACTCCAGCGGCACCAGCCAGA; and to aspartic acid - Forward primer 5' TTGGGCCAGACTCCAGCGGACCCAGCCAGA, and the reverse primer

5' CGCTGGAGTCTGGCCCAAGTATGAAGACGG. The mutations were confirmed by sequencing. The double mutants were made using the one of the previously made mutants as a template.

HPV 18E6: The HA-tagged HPV18E6 plasmid used has been described previously (Massimi et al 2006).

PCMV Flag-Ubiquitin: Was a kind gift from Dr. Michael Ehlers, Duke University, North Carolina, USA.

EBG2T GST- Dlg, S158A and S209A: Were kind gifts from Dr. Ana Cuenda, University of Dundee.

PGEX 2T Dlg: The above sequences were also cloned into the PGEX 2T plasmid to be expressed as Glutathione S-transferase fusion proteins, by partial BamHI - EcoRI digestions of the original PCDNA3 plasmid.

Antibodies.

The following commercial antibodies were used at dilutions indicated: Mouse monoclonal antibody against Dlg (IF 1:200, WB 1:500), anti-Scrib goat polyclonal antibody (IF 1:200) and anti-Dlg polyclonal antibody (IF 1:100, WB 1:500) were purchased from Santa Cruz. Anti-HA monoclonal antibody 12CA5 (Roche; WB 1:100, IF 1:100) anti-β-galactosidase β-gal (Promega, WB 1:5000), polyclonal rabbit anti-α-actin (Sigma 1:1000), polyclonal rabbit anti-HA Y-11 (Santa Cruz, IF 1:100), anti-Flag mouse monoclonal antibody M2 (Sigma, WB 1:2000), anti-cyclin B mouse monoclonal antibody (Calbiochem, WB 1:500), p53 mouse monoclonal antibody (DO-1, Santa Cruz 1:500), anti-γ-tubulin mouse monoclonal antibody (Sigma, WB 1:5000), anti-α-tubulin mouse monoclonal antibody (Abcam, WB 1:1000), anti-p84 mouse monoclonal antibody (Abcam, WB 1:1000), anti-GST mouse monoclonal antibody (Calbiochem, WB 1:500), anti-Transferrin mouse monoclonal antibody (Abcam, IF 1:200), anti-lamin A/C mouse monoclonal antibody

(Abcam, WB 1:1000). The anti-Hugl-1 mouse monoclonal antibody was a kind gift from Dr. Dennis Strand.

Phospho-specific Antibodies.

Phospho-specific antibodies were designed against Serines 158 and 442 on hDlg using the peptide sequences shshi(S)pikpte (for serine 158), and lgqtpa(S)parysp (for serine 442), and produced in rabbits by Biosense S.r.l for Eurogentec limited, Milano, Italy. They were used at a dilution of 1:1000 in western blots. Appropriate secondary antibodies conjugated to HRP were purchased from DAKO and used for western blotting at a dilution of 1:2000.

Fusion protein purification

GST-tagged proteins were expressed and purified as described previously (Thomas et al, 1996). Briefly, 40ml of an overnight culture of *E.coli* strain DH5- α previously transformed with the appropriate expression plasmids were inoculated in a one to ten volume of Luria Broth (LB) containing ampicillin and grown at 37°C upto an OD of 0.6 at 395nm. Recombinant protein expression was induced for 3hrs with 1nM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication in lysis buffer (PBS, 1% Triton X-100, 100 U/ml DNAse, protease inhibitors cocktail, Calbiochem) and the lysates were then cleared from cell debris by centrifugation. The GST-fusion proteins were then incubated for 1hr with glutathione-conjugated agarose. The mixture was then washed several times with the lysis buffer by centrifugation and the purity of the fusion protein was determined by SDS-PAGE and Coomassie Brilliant Blue R (Sigma) staining.

Immunoprecipitation and Western Blotting.

Total cellular extracts were prepared by directly lysing cells from 6-well dishes in SDS loading buffer. Alternately cells were lysed in either E1A buffer (25mM HEPES pH 7.0, 0.1%NP-40, 150mM NaCl, plus protease inhibitor cocktail I; Calbiochem) or RIPA buffer (50mM Tris HCl pH 7.4, 1%NP-40, 150mM NaCl, 1mM EDTA, plus protease inhibitor cocktail I; Calbiochem). After incubation on ice for 20min, lysates were cleared by centrifugation at 13000rpm for 10min. The supernatant (soluble fraction) and pellet (insoluble fraction) were analysed by SDS-PAGE and Western blotting. For Western blotting, 0.45µm nitrocellulose membrane (Schleicher & Schuell) were used and membranes were blocked for 1hr at 37°C in 10% milk/PBS followed by the incubation with the appropriate primary antibody diluted in 10% milk/0.5%Tween 20 for 2hrs. After several washings with PBS 0.5%Tween 20, secondary antibodies conjugated with HRP (DAKO) were diluted in 1%milk/0.5%Tween 20 and incubated for 1hr. Blots were developed using Amersham ECL technique according to the manufacturer's instructions. In the case of Western blotting for phospho-specific Dlg, the blots were blocked as mentioned above and then incubated with the respective phospho-specific antibodies in 5%BSA/0.5%Tween 20, overnight at 4°C. All western blotting experiments were repeated thrice for verification and were quantified by densitometry where indicated using Adobe Photoshop.

Half-life experiments.

293 cells were transfected using calcium phosphate precipitation with 2µg of wild type HA-Dlg or the mutants. 24hrs after transfection, cells were treated for different time points as indicated with cycloheximide (50µg/ml in DMSO) to block protein synthesis. Total cellular extracts were then analysed by

Western blot and the intensity of the bands on the X-ray film was measured using Adobe Photoshop.

Immunofluorescence and Microscopy.

Cells were stained *and fixed for im*munofluorescence as described previously (Grm et al, 2005). Briefly, cells were fixed with 3.7% paraformaldehyde in PBS for 20min and permeabilised with 0.1% Triton X-100 in PBS for 5min. Primary antibodies were incubated for 2hr at 37°C, followed by extensive washing in PBS and incubation for 30min at 37°C with secondary anti-rabbit or anti-mouse conjugated with fluorescein or rhodamine (Molecular Probes). For the visualisation of the nucleus, cells were stained in 1µM Hoechst (Sigma No. 33258; bisBenzimide). Samples were then washed several times with water and mounted using Vectashield mounting medium (Vector Laboratories Inc.) on glass slides.

Slides were analysed using either a Leica DMLB fluorescence microscope equipped with a Leica photo camera (A01M871016) or a Zeiss LSM 510 confocal microscope and the data were collected utilising the X100 objective oil immersion lens. To avoid cross talk between channels during confocal scanning the following settings were used: FITC was excited with a 488nm line of an Argon laser at which the excitation of rhodamine is negligible; rhodamine was excited with a 543nm line of a Neon laser at which the excitation of FITC is negligible. The FITC emission was monitored using a 505-530nm filter and the rhodamine was monitored using a 560nm longpath filter.

Cell Cycle Synchronisation.

To synchronise HaCaT cells, aphidicolin (Sigma) was added at a concentration of 4µg/ml to asynchronous growing cells for 24hrs. The aphidicolin-

containing medium was then removed and the cells washed in 10ml of PBS thrice. The PBS was then replaced with complete medium and the cell synchronisation checked by FACS analysis. Cells were harvested at different times (including time point 0 for G1 phase) and DNA content was assessed by propidium iodide staining and FACS analysis as described previously (Banks et al, 1990).

In vivo degradation assays.

293 cells were transfected with 2µg of HA-Dlg or mutant plasmids, and varying quantities (3µg and 6µg) of the PCD HPV18 E6 plasmid, along with 0.15µg of LacZ expression plasmid. 24hrs post transfection, the cells were harvested and analysed by Western blotting.

Ubiquitination Assays.

293 cells were transfected with 2µg of HA-Dlg or mutant plasmids, and 0.5µg of PCMV-Flag-Ubiquitin. 24 hrs post transfection, the cells were treated with proteasome inhibitors CBZ (MG132, Sigma, 50µM) and LLnL (Sigma, 50µM) for 3 hrs, after which E1A extraction was performed and the soluble fraction was incubated with anti-HA agarose beads (Sigma) to pull down Dlg for 1-2hrs on a rotating wheel at 4°C. The agarose beads were then extensively washed and the precipitated proteins were analysed by Western blotting using the Flag antibody (M2, Sigma) to detect Ubiquitin.

Subcellular Fractionation Assays.

Differential extraction of HaCaT cells to obtain four different fractions - F1 - Cytosol, F2 - Organelles and Membrane, F3 - Nuclear and F4 - Cytoskeleton - was performed using the Calbiochem ProteoExtract Fractionation Kit. Cells were grown to 90% confluency and then treated with either sorbitol, MG-132

or Sorbitol and MG-132. The procedure followed thereafter was according to the manufacturer's instructions.

Nuclear Fractionation Assays.

Nuclear fractionation was performed on various cell lines grown to a 90% confluence on 10cm dishes. The procedure followed was according to the manufacturer's instructions, using the NE-PER Nuclear and Cytoplasmic Extraction Reagents from Pierce Biotechnology (# 78833).

Inhibitors.

The following inhibitors were dissolved in DMSO and used at the indicated concentrations:

Nocodazole (Sigma, 2μM), Aphidicolin (Sigma, 5μM), CBZ (MG132 Sigma, 50μM), Sodium Monensin (Calbiochem, 5μM).

Kinase Inhibitors.

Roscovitine - active against cdk1 and cdk2 (Calbiochem 50μM), ERK1/2 inhibitor (PD98059, Sigma 10μM), p38alpha/beta inhibitor (SB203580, Sigma 20μM), JNK inhibitor (SP600125, Sigma 10μM).

In vitro kinase assays.

Purified GST fusion proteins were incubated with commercially purified CDK2-cyclin A kinase, cdc2- cyclin B kinase, GSK3beta kinase, CKI kinase (New England Biolabs) or CKII kinase (Promega) for 20min at 30°C in phosphorylation buffer (0.25M Tris pH 7.5, 1M $MgCl_2$, 3M NaCl, 0.3mM aprotinin, 1mM Pepstatin) supplemented with 56nM [^{32}P] γ-ATP (Perkin Elmer) and 10mM ATP. After extensive washing, the phosphorylated proteins were monitored by SDS-PAGE and autoradiography.

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